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Identification of dipeptidyl peptidase 3 as the Angiotensin-(1–7) degrading peptidase in human HK-2 renal epithelial cells

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Abstract

Angiotensin-(1–7) (Ang-(1–7)) is expressed within the kidney and exhibits renoprotective actions that antagonize the inflammatory, fibrotic and pro-oxidant effects of the Ang II-AT1 receptor axis. We previously identified a peptidase activity from sheep brain, proximal tubules and human HK-2 proximal tubule cells that metabolized Ang-(1–7); thus, the present study isolated and identified the Ang-(1–7) peptidase. Utilizing ion exchange and hydrophobic interaction chromatography, a single 80 kDa protein band on SDS-PAGE was purified from HK-2 cells. The 80 kDa band was excised, the tryptic digest peptides analyzed by LC-MS and a protein was identified as the enzyme dipeptidyl peptidase 3 (DPP 3, EC: 3.4.14.4). A human DPP 3 antibody identified a single 80 kDa band in the purified enzyme preparation identical to recombinant human DPP 3. Both the purified Ang-(1–7) peptidase and DPP 3 exhibited an identical hydrolysis profile of Ang-(1–7) and both activities were abolished by the metallopeptidase inhibitor JMV-390. DPP 3 sequentially hydrolyzed Ang-(1–7) to Ang-(3–7) and rapidly converted Ang-(3–7) to Ang-(5–7). Kinetic analysis revealed that Ang-(3–7) was hydrolyzed at a greater rate than Ang-(1–7) [17.9 vs. 5.5 nmol/min/μg protein], and the Km for Ang-(3–7) was lower than Ang-(1–7) [3 vs. 12 μM]. Finally, chronic treatment of the HK-2 cells with 20 nM JMV-390 reduced intracellular DPP 3 activity and tended to augment the cellular levels of Ang-(1–7). We conclude that DPP 3 may influence the cellular expression of Ang-(1–7) and potentially reflect a therapeutic target to augment the actions of the peptide.

Keywords

Renin angiotensin system; Angiotensin-(1; 7); HK-2 cells; DPP 3

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1. Introduction

We recently identified a peptidase activity in the brain medulla and cerebrospinal fluid (CSF) of sheep that metabolized angiotensin-(1–7) [Ang-(1–7)] at low picomolar concentrations [8,13-15]. Marshall et al. [15], using a partially purified fraction of the peptidase from brain medulla, showed the enzyme exhibited a very high affinity (IC_{50} < 1 nM) to the metallopeptidase agent JMV-390, but was insensitive to other metallopeptidase agents that inhibit neprilysin (NEP, EC. 3.4.24.11), neurolysin (EC 3.4.24.16) and thimet oligopeptidase (TOP, EC3.4.24.15) [15]. The enriched peptidase hydrolyzed Ang-(1–7) at a 12-fold higher rate than Ang II, but failed to metabolize the larger biologically active peptides apelin 13, bradykinin and neurotensin [15]. Interestingly, the Ang-(1–7) peptidase activity was 3-fold higher in the CSF of glucocorticoid-exposed sheep, an experimental model of *in utero* fetal programing that exhibits higher blood pressure, baroreceptor dysfunction and lower expression of the AT_7/Mas receptor [14]. The peptidase activity negatively correlated with endogenous levels of Ang-(1–7) in the CSF. Therefore, central expression of an Ang-(1–7) peptidase may potentially regulate Ang-(1–7) tone within key cardiovascular centers of the brain to influence blood pressure and baroreflex function [8].

Although we originally identified Ang-(1–7) as an endogenous component of the brain renin-angiotensin system (RAS) over 25 years ago [7], the peptide and its receptor Mas were subsequently shown to be expressed within the kidney, as well as other peripheral tissues [5,20]. In contrast to the Ang II-AT1 receptor pathway, Ang-(1–7) increases renal blood flow and acts directly as a natriuretic to enhance sodium excretion likely through the stimulation of nitric oxide [5]. Ang-(1–7) also exhibits renoprotective actions and antagonizes the inflammatory, fibrotic and pro-oxidant actions of Ang II [4,6,21]. Indeed, Ang-(1–7) treatment was recently shown to be superior to AT1 receptor antagonists to attenuate the progression of diabetic renal damage and advanced glycation end product (AGE)-induced myofibroblast transition and cellular hypertrophy [3,28]. The proximal tubule of the kidney is considered a key site for an intrarenal RAS expressing the biosynthetic components angiotensinogen, renin, and ACE that participate in the local formation of Ang II [17]. The tubules also express the ACE homolog ACE2 that efficiently converts Ang II to Ang-(1–7), as well as the endopeptidases NEP and TOP that process Ang I directly to Ang-(1–7) [6,27]. Moreover, the Ang-(1–7) peptidase activity is expressed in the sheep renal cortex, isolated proximal tubules and in human HK-2 proximal tubule cells [25]. The Ang-(1–7) peptidase constituted the sole enzymatic activity in the HK-2 cells that metabolized Ang-(1–7) [25]. Therefore, the current study utilized the human HK-2 cells as a source of the Ang-(1–7) degrading activity to purify, identify and characterize the peptidase. Following extensive purification, a single protein band revealed on SDS-PAGE was analyzed by LC-MS and the enzyme dipeptidyl peptidase 3 (DPP 3, EC: 3.4.14.4) was identified in the HK-2 cell line.

2. Methods

2.1. Cell culture

Human HK-2 cells derived from human proximal tubular cells were obtained from ATCC (Manassas, VA, USA). The cells were incubated at 37°C under 5% CO2 humidified atmosphere, and were routinely maintained in DMEM/F12 supplemented with 10% FBS,

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insulin-transferrin-selenium-cortisol, 100μg/mL penicillin and 100 μg/mL streptomycin [26]. HK-2 cells were placed in serum-free DMEM/F12 media for 24 h prior to the metabolism and purification studies.

2.2. Cell preparation

Frozen cell pellets were resuspended in HEPES buffer (25 mM Na^+ free HEPES, 10 μM $ZnCl_2$, 125 mM NaCl, 0.01% Triton, pH 7.4) and sonicated briefly utilizing an ultrasonic processor (Model W-380 Heat Systems-Ultrasonics, Inc.) and centrifuged at 100,000 ×g for 30min at 4°C. Supernatants were stored on ice and utilized for peptide metabolism experiments or the enzyme purification (HK-2 supernatant).

2.3. HK-2 peptidase purification

The Ang-(1–7) peptidase was purified from HK-2 cells using a similar approach to enrich the brain peptidase but with an additional column step and SDS-PAGE fractionation (Fig. 1) [15]. The HK-2 supernatant was initially pooled and concentrated on a 30 kDa cut-off filter (Millipore, Bedford, MA, USA). The concentrated supernatant was resuspended in a final volume of 5 mL HEPES buffer (25 mM HEPES, 50 mM NaCl, 10 μM $ZnCl_2$, 0.01% Triton, pH 8.0) and applied to a Cibacron Blue Sepharose Fast Flow (GE Healthcare Bio-Sciences, USA) column (1 × 5 cm) equilibrated in the same HEPES buffer. The flow-through fraction was applied directly to a diethylaminoethyl Sepharose column (DEAE, 1 × 10 cm, Sigma-Aldrich, St. Louis, MO, USA). The column was subsequently washed in HEPES buffer with an increasing step gradient of NaCl (75, 100, 125,250 mM). The eluted activity in the 125 and 250 mM NaCl fractions were diluted in HEPES buffer without NaCl then applied to a Q-Sepharose Fast Flow column (1 × 10 cm, Sigma-Aldrich) with a step gradient of increasing NaCl (155,185, 215, and 250 mM). Peptidase activity eluted at 185–215 mM NaCl and the two fractions were concentrated in the HEPES buffer containing 1 M NH_4SO_4 and applied to 2 × 10 cm Phenyl-Sepharose column (Sigma-Aldrich). This column was washed with 1 M NH_4SO_4 and a decreasing step gradient was applied (500, 250, 100, 0mM). The enzyme activity primarily eluted in 250 mM NH_4SO_4 and the fraction was concentrated on a 30 kDa filter in the HEPES buffer to remove the NH_4SO_4.

2.4. Native and SDS polyacrylamide electrophoresis

The purified peptidase from the Phenyl Sepharose column was fractionated by both native and SDS 10% polyacrylamide gels for 1hat120Vin Tris-glycine. Gels were stained with the protein dye Imperial Blue and the sole band at 80 kDa excised for peptidase activity (native gel) or the SDS gel for MS analysis (MS Bioworks, Ann Arbor MI USA). For Western blot analysis of the purified enzyme and the human DPP3 standard (R&D Systems, Minneapolis MN USA), the SDS gels were transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% Bio-Rad Dry Milk (Bio-Rad, Hercules, CA, USA) and Tris buffered saline (TBS) with Tween (0.05%) and probed overnight at 4 °C with a human DPP 3 primary antibody (1:10,000; Abcam, Cambridge MA USA). Membranes were treated with horseradish peroxidase (HRP)-labeled polyclonal anti rabbit secondary antibodies (1:5000) for 1 h and detected with chemiluminescent substrates (Pierce Biotechnology, Rockford, Illinois, USA).

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2.5. Peptidase assays

Metabolism reactions were conducted at 37°C in the HEPES assay buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, 0.01% Triton, pH 7.4) in the HK-2 cytosol, the column fractions, the supernatant from the excised band of the native gel and human DPP 3. The assay contained a final concentration 0.5 nM ^{125}I-Ang-(1–7) and 100nM Ang-(1–7) in a volume of 250 μL. The reaction was terminated after 60min by addition of ice-cold 1.0% phosphoric acid. The supernatants were filtered (0.2 μm) prior to separation on a Shimadzu Prominence HPLC equipped with an Aeris Peptide XB-C18 (2.1 × 100 mm, Phenomenex, Torrance, CA, USA). The ^{125}I-products were quantified using a Bioscan flow-through γ detector and activity were expressed as fmol of ^{125}I-Ang-(3-4) formed per minute per mg protein (fmol/min/mg) [13].

For the kinetic studies, we varied the concentration of unlabeled Ang-(1–7) or Ang-(3–7) from 0 to 50 μM in the presence of 0.5 nM ^{125}I-Ang-(1–7)or^{125}I-Ang-(3–7).The reaction conditions were 1 ng DPP 3 for 15 min for Ang-(3–7) and 5 ng for 60 min for Ang-(1–7) at 37 °C. The generation of ^{125}I-Ang-(3–4) was determined following HPLC and the Bioscan flow-through γ detector.

For the metabolism of unlabeled Ang-(1–7)by DPP 3, 100 μM of Ang-(1–7) was incubated with 100 ng of human DPP 3 for 60 min at 37 °C with or without the JMV-390 inhibitor (1 μM, Tocris Bioscience, Bristol, UK) under the assay conditions described above. In this case, the HPLC profile of peptide products was monitored at 220 nM using an inline spectrophotometer.

2.6. Cell inhibitor studies

To establish the effect of peptidase inhibition on the cellular expression of Ang-(1–7), the HK-2 cells were placed in serum-free DMEM/F12 media for 24 h and then treated with 20 or 200 nM JMV-390 every 24 h for 3 days. Cell plates were placed on ice, rinsed in cold PBS, scraped and stored at -80 °C. The cell pellet was brought up to 5 mL of Milli-Q water and immediately placed in a boiling water bath for 15min. The solution was acidified with 0.1% formic acid, sonicated and then centrifuged at 25,000 × g (30min at 4°C). The supernatant was applied to a Strata-X (Phenomenex, CA, USA) SPE reversed phase extraction column. The column was washed with Milli-Q water and the peptide fraction eluted in 90% methanol with 0.1% formic acid. The eluent was evaporated in a Savant vacuum centrifuge, reconstituted in the Ang-(1–7) RIA buffer and peptide content determined. As previously described, the Ang-(1–7) RIA recognizes Ang-(2–7) and Ang-(3–7), but does not recognize (<0.01%) other angiotensin peptides including Ang II and AngI [27]. The Ang-(1–7) RIA exhibits a sensitivity of 2.5 fmol per tube. The peptide content of Ang-(1–7) in the HK-2 cells was expressed as fmol per mg protein of the cell extract.

2.7. Statistics

Data are expressed as mean±SEM. A Student's t-test and one-way ANOVA with Bonferroni posttest were applied for the statistical analysis of the data (GraphPad Prism 6, San Diego, CA USA). The criterion for statistical significance was set at $p<0.05$. The inhibitory constants (IC_{50}) for the peptidase activity were determined by non-linear regression one-site

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competition with no constraints (GraphPad Prism 6). The Michealis-Menten constants (Km, Vmax) for DPP 3 hydrolysis of Ang-(1–7) and Ang-(3–7) were derived in Prism 6.

3. Results

As shown in the purification outline (Fig. 1), the 100,000 × g cytosolic fraction from the HK-2 cells was initially concentrated on a 30 kDa cut-off filter and applied to a Cibacron Blue 3AG column. The peptidase activity was not retained and the flow through was immediately applied to DEAE Sepharose; Ang-(1–7) degrading activity primarily eluted in 125/250 mM NaCl fractions. These fractions were diluted in HEPES buffer and applied to a Mono-Q ion exchange column, washed and the majority of peptidase activity eluted in the 185/215 mM NaCl fractions. The Q fractions were concentrated, the buffer adjusted to 1 M NH4SO4 and applied to a Phenyl-Sepharose column. The peptidase activity was retained on the column under the 1 M NH4SO4 loading conditions and a reverse step gradient was applied; the activity eluted in the 250 mM NH4SO4 fraction. This fraction was concentrated on a 30 kDa filter and analyzed by SDS-PAGE stained with Imperial Blue. As shown in Fig. 2A, the full-length SDS gel reveals a single protein band at ~80 kDa for the Phenyl-Sepharose fraction. We excised the 80 kDa band from the SDS gel and submitted it for MALDI-MS analysis. The MS analysis revealed 8 human proteins that included keratin, heat shock protein 70, and DPP 3, as well as bovine serum albumin (BSA) that likely reflects internalization from fetal bovine serum by the tubule cells (Fig. 2B). Of the proteins identified, only DPP 3 exhibited the expected molecular size and proteolytic activity to hydrolyze Ang-(1–7). We then obtained an antibody to human DPP 3 and the recombinant human enzyme to evaluate immunostaining of the purified Ang-(1–7) peptidase and human DPP 3. As shown in Fig. 2C, the full-length immunoblot reveals an identical band at 80 kDa for human DPP 3 (lanes 1-2) and the purified Ang-(1–7) peptidase (lanes 3-4) from the HK-2 cells. Although not shown, the Phenyl Sepharose fraction was also analyzed by native PAGE and the stained gel revealed a single band at 80 kDa. This band contained peptidase activity that metabolized ^{125}I-Ang-(1–7) to ^{125}I-Ang-(3-4) (Fig. 2D).

The metabolism of ^{125}I-Ang-(1–7) by the purified Ang-(1–7) peptidase and human DPP 3 was then compared (Fig. 3). As shown in the chromatographs, both the Ang-(1–7) peptidase and DPP 3 cleaved ^{125}I-Ang-(1–7) to predominantly ^{125}I-Ang-(3-4) (Fig. 3A and B). Addition of increasing concentrations of JMV-390 attenuated the metabolism of ^{125}I-Ang-(1–7) by the Ang-(1–7) peptidase (Fig. 3C and 3E) and by DPP 3 (Fig. 3D and 3F). The inhibition studies of JMV-390 for ^{125}I-Ang-(1–7) hydrolysis revealed an IC50 of 0.20 ± 0.01 nM for the Ang-(1–7) peptidase and 1.40 ± 0.01 nM for DPP 3 (Fig. 3G). We confirmed that DPP 3 also hydrolyzed unlabeled Ang-(1–7). As shown in the chromatograph (Fig. 4B), incubation of Ang-(1–7) with DPP 3 for 60min at 37°C revealed a major peak corresponding to the metabolite Ang-(5–7). Treatment of DPP 3 with the JMV-390 inhibitor essentially abolished the metabolism of Ang-(1–7) to Ang-(5–7) (Fig. 4C). Note that unlabeled Ang-(1–7) and Ang-(3–7) co-elute under the current HPLC-UV conditions while their ^{125}I-forms are distinguished (Fig. 3). Addition of the ^{125}I moiety results in greater retention of Ang-(3–7) than Ang-(1–7) such that ^{125}I-Ang-(3–7) elutes approximately 3 min after ^{125}I-Ang-(1–7) (Figs. 2 and 3). Moreover, the HPLC-γ detection of ^{125}I-peptides identifies only their ^{125}I-

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Tyr residue and as the Ang-(5–7) metabolite lacks the Tyr residue, this peak is not detected in the chromatographs from Fig. 3.

DPP 3 is a metallothiolpeptidase that cleaves two amino acids from the N-terminus of peptide substrates 4-8 residues in length [1].Thus, the predicted pattern of Ang-(1–7) metabolism by DPP 3 should be the immediate formation of the pentapeptide Ang-(3–7) from the initial hydrolysis of the Arg^2-Val^3 bond and the subsequent formation of the tripeptide Ang-(5–7) by hydrolysis of the Tyr^4-Ile^4 bond; there should be no further metabolism as DPP 3 does not cleave peptides of 3 or less residues [1]. However, based on the expected metabolism, it was unclear why we failed to observe a predominant peak of ^{125}I-Ang-(3–7). Therefore, we performed a kinetic analysis of ^{125}I-Ang-(1–7) and ^{125}I-Ang-(3–7) metabolism by human DPP 3. For these studies, we used a fixed concentration of the ^{125}I-substrate and increasing concentrations of the unlabeled substrate assuming the peptidase equally recognizes both labeled and unlabeled peptides. Shown in Fig. 5 are the saturation curves for the hydrolysis of Ang-(1–7) and Ang-(3–7) by DPP 3. Derivation of the Michealis-Menten constants revealed a higher Km value of 12 μM for Ang-(1–7) as compared to a Km of 3 μM for Ang-(3–7) (Table 1). Moreover, the Vmax value for Ang-(3–7) was 3-fold higher than that for Ang-(1–7) [17.9 vs 5.5 nmol/min/μg] (Table 1). Calculation of the specificity constant (kcat/Km) also revealed a higher value for Ang-(3–7) than Ang-(1–7) [7.9 vs 0.6] (Table 1). These data likely explain the absence of a peak for Ang-(3–7) as the pentapeptide is rapidly degraded to Ang-(5–7) following the initial hydrolysis of Ang-(1–7) (Fig. 5, inset).

Finally, we sought to address whether DPP 3 influences the intracellular expression of Ang-(1–7) in the HK-2 cells by chronically treating the cells with JMV-390. Cells were exposed to 20 and 200 nM JMV-390 for 72 h; the chosen doses of JMV-390 were 100-1000-fold higher than that of the IC50 (0.2 nM) for the purified Ang-(1–7) peptidase. As shown in Fig. 6A, basal or control peptidase activity was 136±6 fmol/min/mg (n = 3). Treatment with 20nM and 200 nM JMV reduced activity by 35% (76 ±13 fmol/min/mg, n = 2) and 85% (17±3 fmol/min/mg; $p<0.05$, n = 3), respectively. In Fig. 6B, basal content of Ang-(1–7) in the HK-2 cells was 22 ±2 fmol/mg protein (n = 3). The 20 nM JMV-390 dose increased Ang-(1–7) by 68% (37 ±5 fmol/mg protein, n = 3); however, the increase did not achieve statistical significance (Fig. 6B). In contrast, treatment with 200 nM JMV-390 significantly lowered Ang-(1–7) levels (15 ±3 fmols/mg protein; $p<0.05$, n = 3) as compared to 20 nM JMV-390 (Fig. 6B).

4. Discussion

Current evidence strongly supports an alternative Ang-(1–7) axis of the RAS in the circulation, kidney and other tissues that may buffer or antagonize the actions of the Ang II-AT1 receptor axis [4,5,8,20]. Enzymes that form Ang-(1–7) directly from Ang I *in vivo* include the endopeptidases neprilysin and thimet oligopep-tidase while ACE2 and prolyl carboxypeptidase hydrolyze Ang II to Ang-(1–7) [6,26]. In regards to the metabolism of Ang-(1–7), we identified ACE as a primary pathway in the circulation that degrades Ang-(1–7) [6]. Circulating levels of Ang-(1–7) markedly increase following administration of ACE inhibitors that reflect the reduced metabolism of Ang-(1–7), as well as the shunting of Ang I

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away from Ang II and towards Ang-(1–7)-forming pathways [6]. Indeed, Marshall et al. [8,15] demonstrated ACE activity in the sheep CSF that hydrolyzed Ang-(1–7); however, these studies also revealed another peptidase activity that metabolized Ang-(1–7) to a greater extent than ACE. Moreover, this Ang-(1–7)-degrading activity was 3-fold higher in the sheep exposed to antenatal betamethasone, an experimental model of fetal programming that exhibits hypertension and reduced baroreflex sensitivity [8]. Wilson et al. [25] identified a similar if not identical peptidase activity that degraded Ang-(1–7) in the sheep proximal tubules and the human HK-2 cell line. As this activity was the sole pathway for the metabolism of Ang-(1–7) in the HK-2 cells, we isolated the Ang-(1–7) peptidase activity from these cells by multiple chromatographic steps and SDS-PAGE and identified the enzyme as DPP 3. DPP 3 is a soluble metallopeptidase that is widely distributed in both central and peripheral tissues [18]. The enzyme cleaves 2 residues at a time from the N-terminus of various peptides with an optimal substrate length of 4-8 amino acids [1]. The fact that DPP 3 is a thiol metallopeptidase explains the unusual sensitivity of the enzyme to the thiol inhibitors PCMB and APMA, as well as the chelating agents ophenanthroline and EDTA [2,15]. We reported that the Ang-(1–7) peptidase in brain and renal tubules exhibited a low IC_{50} (~1 nM) to the metallopeptidase inhibitor JMV-390, and the recombinant form of DPP 3 was also potently inhibited by the JMV compound (IC50 of ~1 nM) [15,25].

Although we originally considered the Ang-(1–7) degrading activity to be an endopeptidase that forms Ang-(1-4), the present studies confirm that the DPP 3 hydrolyzes Ang-(1–7) to the pentapeptide Ang-(3–7) which undergoes rapid conversion to Ang-(5–7) [15,25]. Indeed, using ^{125}I-Ang-(1–7) as the peptide substrate, accumulation of ^{125}I-Ang-(3–7) was not evident. Moreover, kinetic studies on the hydrolysis of Ang-(1–7) and Ang-(3–7) by DPP 3 revealed that Ang-(3–7) was a superior substrate. The kcat for the hydrolysis of Ang-(3–7) by DPP 3 was 3-fold higher than Ang-(1–7) while the Km for Ang-(3–7) was 6-fold lower than that for Ang-(1–7); the overall kcat/Km was 12-fold higher for Ang-(3–7) and this likely explains the apparent absence of an Ang-(3–7) peak following the initial hydrolysis of Ang-(1–7). These data are also consistent with our previous study in which the fluorescently quenched Ang-(1–7) analog [Abz-Asp^1, Tyr $(NO_2)^7$]-Ang-(1–7)] was resistant to the HK-2 peptidase [25]. Peptide substrates that contain a N-terminally blocked group are likely resistant to hydrolysis by DPP 3 [18].

DPP 3 is primarily an intracellular peptidase, although we detected activity in the cell media of the HK-2 cells suggesting the possible secretion or release of the enzyme [25]. The HK-2 cells are reported to express a complete RAS including renin, angiotensinogen, ACE and ACE2; therefore, we assessed whether chronic treatment of the HK-2 cells with the JMV-390 inhibitor would influence the intracellular levels of Ang-(1–7) [21]. We find that the lower dose of JMV-390 reduced the activity of DPP 3 by 35% in the cytosol of the HK-2 cells and increased the cellular levels of Ang-(1–7) approximately 2-fold, although this increase was not statistically significant. The higher dose of JMV-390 markedly lowered DPP 3 activity (>80%), but also reduced Ang-(1–7) content. We previously reported that a high dose of JMV-390 inhibited the direct conversion of ^{125}I-Ang I to ^{125}I-Ang-(1–7) by TOP expressed in the HK-2 cells [25]. It is possible that the higher dose of JMV-390in the current study may have elicited a greater effect to block the generation of Ang-(1–7) rather than attenuate the metabolism of the peptide, thus leading to the lower cellular levels of

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Ang-(1–7). Additional studies are required to determine whether more selective approaches to knockdown DPP 3 would yield a more robust increase in the cellular content of Ang-(1–7), as well as better distinguish the intracellular pathways for the generation and metabolism of the peptide.

Prajapati et al. [19] recently reported that overexpression of DPP 3 and the AT1 receptor in HEK 293 cells attenuated the calcium response to extracellular Ang II. These investigators postulate that DPP 3 degrades intracellular peptide substrates that may influence the signaling of the Ang II-AT_1 receptor complex [19]. It is possible that enhanced degradation of intracellular Ang-(1–7) contributes to the inhibitory actions of DPP 3 on Ang II signaling; however, evidence favors that Ang-(1–7) antagonizes or opposes the actions of the Ang II-AT_1 receptor axis [4,8,20]. Indeed, we would predict that metabolism of Ang-(1–7) by DPP 3 would augment the Ang II response provided the HEK 293 cells have the capacity to generate Ang-(1–7). Others have reported that DPP 3 activity is enhanced in ovarian cancers and that DPP 3 is associated with the aggressiveness of the tumor which may potentially reflect a lower intracellular content of Ang-(1–7) [21-23]. In this regard, Ang-(1–7) exhibits both anti-proliferative and anti-angiogenic actionsto reduce growth in various tumor models [9-12,16,24].

In conclusion, the present studies reveal that the Ang-(1–7) degrading activity in the HK-2 proximal tubule cells is DPP 3 and that the peptidase activity present in the sheep CSF, brain medulla, and proximal tubules is likely DPP 3. The extent that DPP 3 participates in the intracellular expression of Ang-(1–7) in central and peripheral compartments may reflect the co-localization of the peptidase and Ang-(1–7), as well as the presence of other competing peptide substrates for DPP 3 in these tissues. The intracellular pathways involved in the expression of angiotensin peptides are not clearly established; however, elucidation of the role of DPP 3 to regulate Ang-(1–7) may be important to establish the cellular actions of the alternative axis of the RAS.

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Glossary

Ang	Angiotensin
Ang I	[Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}]
Ang II	[Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8]
Ang	(1–7) – [Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7]
Ang	(1-4) – [Asp^1-Arg^2-Val^3-Tyr^4]
Ang	(3–7) – [Val^3-Tyr^4-Ile^5-His^6-Pro^7]
Ang	(5–7) – [Ile^5-His^6-Pro^7]
Ang	(3-4) – [Val^3-Tyr^4]
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
CSF	Cerebrospinal fluid
DPP 3	Dipeptidyl peptidase III
RAS	Renin-angiotensin system

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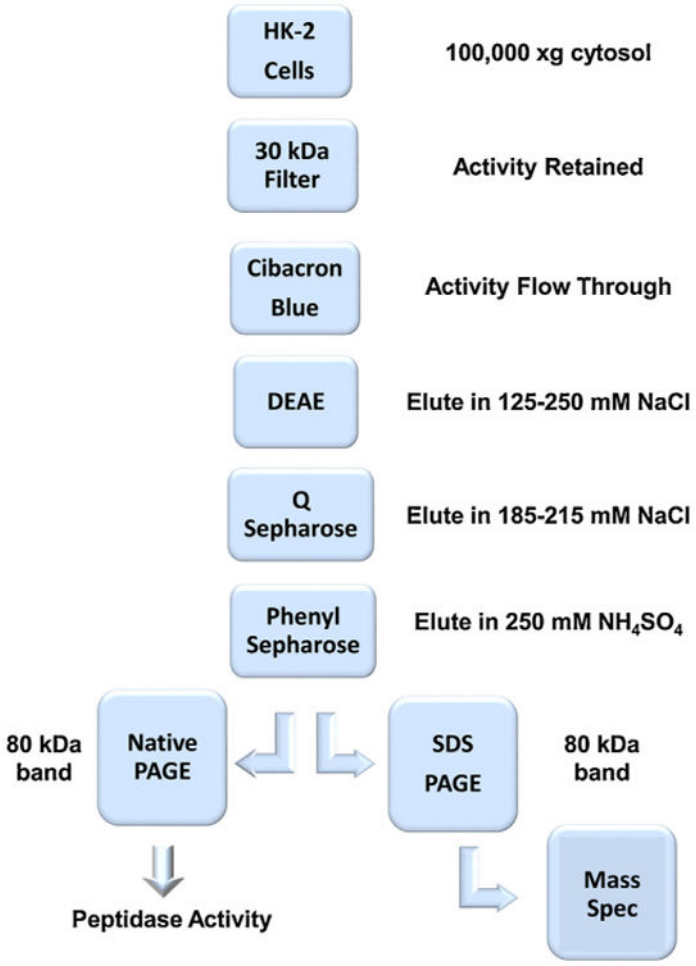

Fig. 1.
Purification scheme for Ang-(1–7) peptidase activity from human HK-2 cells.

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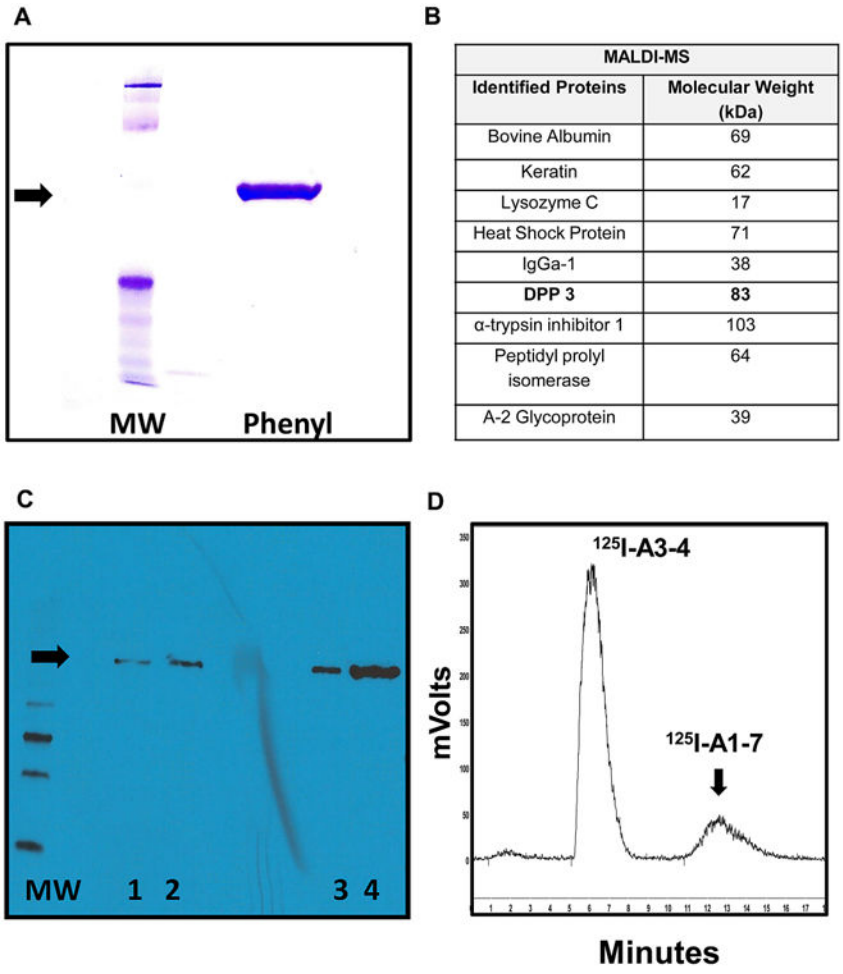

MALDI-MS	
Identified Proteins	**Molecular Weight (kDa)**
Bovine Albumin	69
Keratin	62
Lysozyme C	17
Heat Shock Protein	71
IgGa-1	38
DPP 3	**83**
α-trypsin inhibitor 1	103
Peptidyl prolyl isomerase	64
A-2 Glycoprotein	39

Fig. 2.

Characterization of purified Ang-(1–7) peptidase from HK-2 cells. A: Phenyl column fraction reveals a single 80kDa band (arrow) on SDS-PAGE gel stained with Imperial Blue. B: Proteins identified by MALDI-MS in the 80kDa band excised from SDS-PAGE gel. C: Western blot of human DPP 3 (lanes 1-2) and purified Ang-(1–7) peptidase with human DPP 3 antibody reveals a single 80 kDa band (arrow). D: Excised 80 kDa band from native PAGE gel of purified Ang-(1–7) peptidase from Phenyl column reveals activity that hydrolyzed ^{125}I-Ang-(1–7) to ^{125}I-Ang-(3-4) (panel D). Molecular weight marker, MW.

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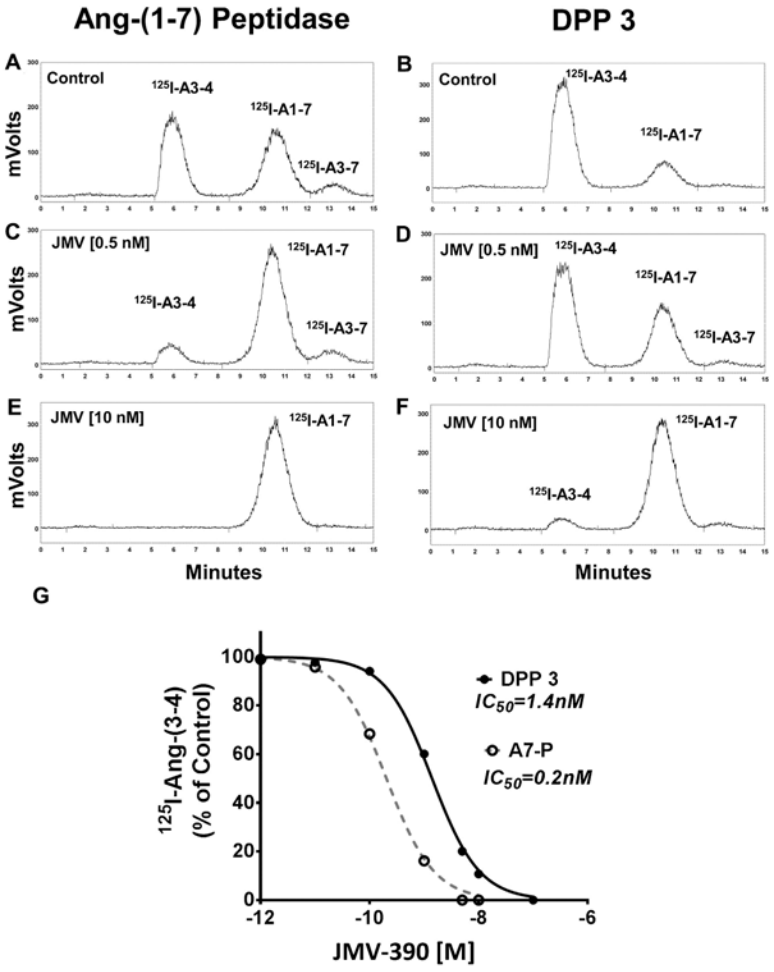

Fig. 3.

Hydrolysis of ^{125}I-Ang-(1–7) by the purified Ang-(1–7) peptidase and human DPP3. Chromatographs show Ang-(1–7) peptidase and DPP 3 hydrolysis of ^{125}I-Ang-(1–7) alone (A and B) or with 0.5 nM JMV-390 (C and D) or with 10nM JMV-390 (E and F). G: Mean values forJMV-390 inhibition of Ang-(1–7) peptidase (A7-P, -○) and DPP 3 (-●-). A monophasic curve fit and IC50 values were derived by Prism 6 (n=3).

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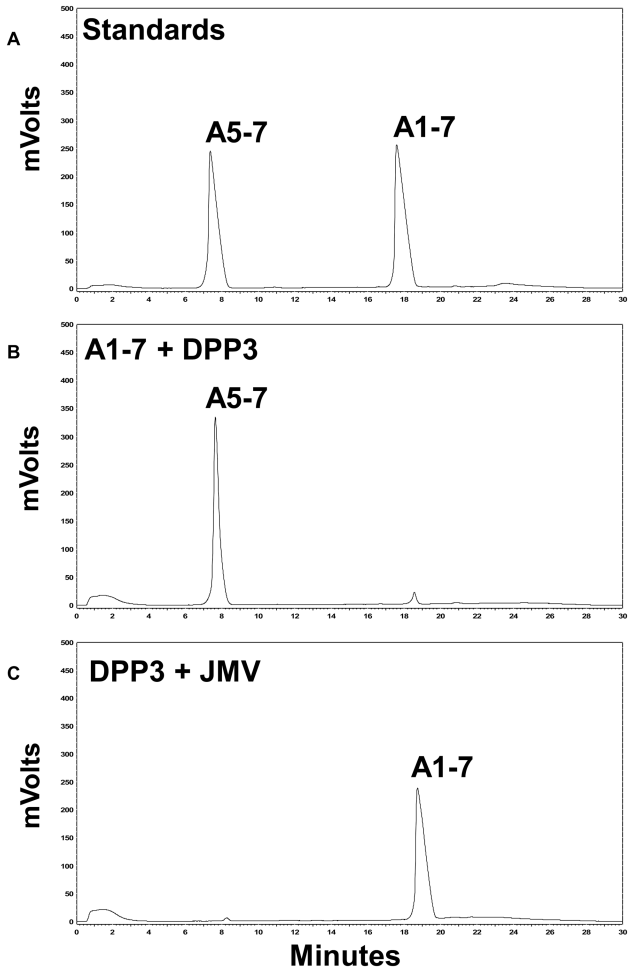

Fig. 4.

Hydrolysis of Ang-(1–7) by human DPP 3. A: HPLC-UVchromatograph of Ang-(5–7) and Ang-(1–7) standards. B: DPP 3 cleaves Ang-(1–7) to the tripeptide Ang-(5–7). C: Pretreatment of DPP 3 withJMV-390 (1 (1 μM) attenuated the metabolism of Ang-(1–7).

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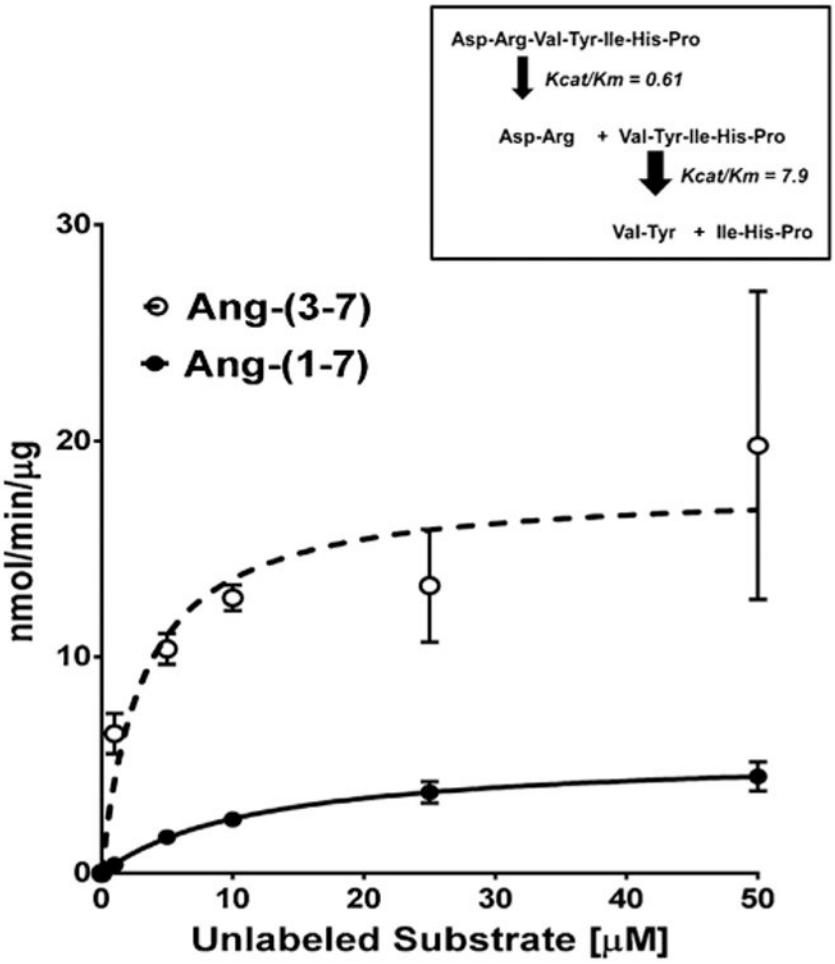

Fig. 5.

Saturation curves forthe hydrolysis of ^{125}I-Ang-(1–7) and ^{125}I-Ang-(3–7) by human DPP 3. Conversion of either ^{125}I-Ang-(1–7) (-ʌ-) or ^{125}I-Ang-(3–7) (-人) to ^{125}I-Ang-(3-4) in the presence of increasing concentrations of unlabeled peptide substrates. Curve fit and kinetic constants were derived by Prism 6 (n=3). ***Inset***: Scheme forthe cleavage of Ang-(1–7) and Ang-(3–7) by DPP 3 based on the kinetic data.

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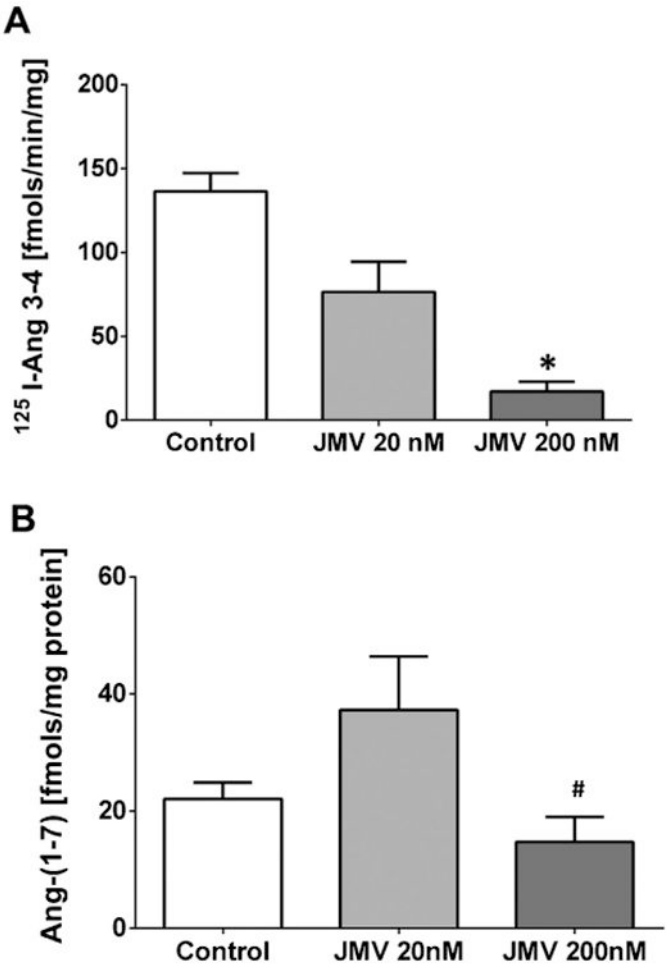

Fig. 6.

Influence of JMV-390 on ^{125}I- Ang-(1–7) metabolism and intracellular Ang-(1–7) content in HK-2 cells. Cells were treated with 20 or 200 nM of JMV-390 for 72h. A: JMV-390 significantly reduced the metabolism of ^{125}I-Ang-(1–7) to ^{125}I-Ang-(3-4) in the 100,000 × g cytosolic fraction of HK-2 cells. Metabolism data are mean±SEM; *p< 0.05 versus Control, n = 3, except for 20 nM JMV dose, n = 2. Statistical analysis by Student's *t*-test (Prism 6). B: The 20 nM dose of JMV-390 increased the intracellular content of Ang-(1–7) in the HK-2 cell extracts while 200 nM JMV-390 significantly decreased the content of Ang-(1–7). Ang-(1–7) content data are mean±SEM;, $^{\#}$p<0.05 versus 20nM JMV, n = 3 all groups. Statistical analysis by one-way ANOVA with Bonferroni post-test (Prism 6).

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Table 1

Kinetic constants for Ang-(1–7) and Ang-(3–7) metabolism by DPP 3.

	Ang-(1–7)	**Ang-(3–7)**
V_{max}	5.5 ± 0.4 nmol/min/μg	17.9 ± 1.8 nmol/min/μg
Km	12±2 μM	3±1 μM
k_{cat}	7.3 s^{-1}	23 8 s^{-1}
k_{cat}/Km	0.61	7.9

Data are mean±SEM, n=3