Evidence for a mitochondrial angiotensin-(1–7) system in the kidney

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THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays a pivotal role in regulating cardiovascular and renal function. Historically, the RAS is characterized as a circulating system that contributes to the regulation of blood pressure and fluid homeostasis. In a paracrine manner, the RAS influences local tissue systems including the brain, adrenal cortex, heart, and kidney. These organ systems were subsequently found to contain a functional RAS capable of responding in an autocrine fashion. Indeed, there is compelling evidence for functional actions of intracellular renin-angiotensin systems in various cell organelles, including the endoplasmic reticulum, nucleus, and the mitochondria within various tissues (1, 2, 4, 5, 25, 26, 42).

Mitochondria are the major energy-producing organelles within cells. These organelles are a primary source of reactive oxygen/nitrogen species and may regulate intracellular redox status and influence cellular signaling. Mitochondrial function and oxidant production are altered in cardiovascular pathologies that may reflect, in part, a role of the ANG II axis of the RAS. ANG II, a potent vasoconstrictor and inflammatory peptide hormone, stimulates the production of mitochondrial reactive oxygen species (ROS) (15, 29). In this regard, recent studies demonstrate an intramitochondrial RAS within a variety of tissue types (1, 5). Abadir et al. (1) report that ANG II type 2 (AT2) receptors are expressed on the inner mitochondrial membrane and are coupled to nitric oxide (NO) production in liver mitochondria. Moreover, an increased ratio of mitochondrial ANG II type 1 (AT1) to AT2 receptors was associated with aging; chronic treatment of aged mice with the AT1 receptor antagonist losartan abrogated the higher AT1 receptor expression (1). These results suggest that one target of chronic RAS blockade to blunt the effects of cellular aging is the mitochondria. Indeed, Ferder, Inversa, and colleagues (11–13) demonstrate that either angiotensin-converting enzyme (ACE) inhibition or angiotensin receptor blockers (ARBs) attenuated the decline in mitochondrial function. However, a more recent study has raised concerns over the biochemical evidence for an intramitochondrial RAS (5). Astin et al. (5) failed to detect RAS components in purified liver mitochondria and reported marginal inhibition of mitochondrial respiration by ANG II at a supraphysiological dose (1 μM), suggesting a nonspecific rather than a direct effect of ANG II.

Since the Abadir and Astin studies focused primarily on the ANG II-AT1/AT2 receptor axis in rodents, we undertook a biochemical analysis of the mitochondrial RAS that examined the ANG-(1–7) axis. The current study provides evidence for several RAS components, including angiotensinogen, active renin, ANG I, and the bioactive peptides ANG II and ANG-(1–7) in purified mitochondria from the sheep renal cortex. Moreover, we demonstrate that purified mitochondria process ANG I to ANG-(1–7) by the endopeptidases nephrilysin and thimet oligopeptidase, suggesting the possibility for an intramitochondrial RAS pathway that forms ANG-(1–7).

METHODS

Animals

Mixed-breed sheep (obtained from a private local vendor) were delivered at term, farm-raised, and weaned at 3 mo of age. Adult male and female sheep (10–12 mo of age) were anesthetized with ketamine and isoflurane and euthanized by exsanguination. The kidneys were removed immediately, and the renal cortex was dissected out on ice for immediate isolation of mitochondria. Cortical tissue or isolated mitochondria were stored at −80°C. All procedures in the current study were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee for animal care.
Isolation of Sheep Renal Cortex Mitochondria

Mitochondria were isolated from fresh sheep renal cortex by a discontinuous Percoll gradient, as described previously (20, 39, 46). The tissue was homogenized in mitochondrial homogenization buffer [75 mM sucrose, 225 mM mannitol, 10 mM Na$^+$-free HEPES, and 0.1 mM EDTA, pH 7.4 (KOH only)] using a Polytron Ultra-Turrax T25 Basic (setting 5), followed by a 50-ml all-glass Dounce homogenizer (Kontes Glass, Vineland, NJ). The homogenate was centrifuged for 5 min at 1,000 g, and then the supernatant was collected and filtered through a 70-μm metal strainer and centrifuged for 10 min at 10,000 g. The resulting pellet, representing the crude mitochondrial fraction, was resuspended in equal amounts of 12% Percoll (GE Healthcare, Buckinghamshire, UK) in Percoll buffer [250 mM sucrose, 0.4 mM EDTA, and 10 mM Na$^+$-free HEPES]. This suspension was layered onto a discontinuous Percoll gradient (24 and 40% in Percoll buffer). The gradient was centrifuged for 5 min at 15,000 g, and the layer between the 24 and 40% Percoll suspensions containing the purified mitochondria was collected. The preparation was washed in Percoll and centrifuged for 10 min at 15,000 g. The purity of the isolated mitochondria was determined by immunoblot analysis (Fig. 1, A–C). Antibodies were obtained from the following sources: voltage-dependent anion channel (VDAC; 1:1,000, Cell Signaling), an outer mitochondrial membrane marker, and several inner mitochondrial markers of the electron transport chain: ATP synthase complex V (CV-ATP5A); ubiquinol-cytochrome c reductase complex II (CII-UCRC2); and succinate dehydrogenase complex subunit B complex II (CII-SDHB) obtained from MitoSciences (1:1,000, Total OXPHOS Antibody Cocktail). A cathepsin B antibody was utilized as a marker for lysosomes and renin granules (50) (1:400, ab58802, Abcam). Membranes were probed with a mouse monoclonal anti-β-actin (1:5,000, Sigma, St. Louis, MO) antibody to assess the degree of purity.

Immunoblot Analysis of RAS Components

Purified mitochondrial homogenates (~100 μg) were boiled in PBS (pH 7.4), diluted in Laemmli buffer with β-mercaptoethanol, separated on 10% SDS polyacrylamide gels for 1 h at 120 V in Tris-glycine SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% Bio-Rad Dry Milk (Bio-Rad, Hercules, CA) and Tris-buffered saline (TBS) with Tween (0.05%) and probed overnight at 4°C with primary antibodies against the following: rat angiotensinogen (internal sequence of angiotensinogen, Total angiotensinogen, residues 42–57, 1:1,000); ANG I sequence of angiotensinogen (ANG I-angiotensinogen, residues 25–34, 1:1,000); renin (1:3,000, Inagami antibody no. 826); renin (1:100, Aviva); prorenin receptor (ATP6IP2; 1:500, Abcam); nephrilysin (NEP; 1:2,000, Cell Signaling); thimet oligopeptidase (TOP; 1:800, Epitomics); and the ANG-(1–7)/Mas receptor protein (MAS; 1:250, Alomone AAR-013). Membranes were treated with horse-radish peroxidase (HRP)-labeled polyclonal anti-rabbit secondary antibodies (1:5,000) for 1 h and detected with ECL chemiluminescent substrates (Advanta, Menlo Park CA).

Renin Assay

Purified mitochondria were isolated as described above and stored at −80°C. The mitochondrial pellet was reconstituted in 1 ml of metabolism buffer (25 mM HEPES, 125 mM NaCl, 10 μM ZnCl$_2$) at pH 7.4, sonicated and left for 60 min on ice. A sample from the homogenate was taken for protein content. For a basal renin activity assay, 400 μg of mitochondrial homogenate was added to 10 μl of nephrectomized sheep plasma as the source of the exogenous angiotensinogen substrate. The assay was performed at 37°C for 4 h in the presence or absence of the renin inhibitor aliskiren (1 μM, final concentration). Aliquots were removed at 30 min, 1, 2, and 4 h and analyzed by Western blotting using the ANG I-angiotensinogen antibody. Activation of prorenin was performed by addition of 5 mg/ml trypsin (Sigma) to the mitochondrial sample for 60 min on ice. Trypsin was inactivated by incubation with an excess of soybean trypsin inhibitor (SBTI; 1 mg/ml, Sigma) for 15 min at room temperature. SBTI is a potent serine protease inhibitor that does not attenuate renin, an aspartyl protease. Following SBTI, the renin assay was performed as described above.

Angiotensin Peptides

Sheep renal cortex mitochondria were isolated as described above and stored at −80°C. The mitochondrial pellet was reconstituted in Milli-Q water and placed in a boiling water bath for 15 min. The mitochondrial fraction was acidified with heptafluorobutyric acid (HFBA) to a final concentration of 0.1%, sonicated, and centrifuged at 20,000 g for 20 min at 4°C. The resultant supernatant was applied to an activated Sep-Pak C$_{18}$ extraction column, washed with 0.1% HFBA, and the peptide fraction was eluted with 3 ml of 100% methanol in 0.1% HFBA. Measurement of immunoreactive ANG I, ANG II, and ANG-(1–7) in the extracted mitochondria was performed using three distinct (RIAs) (4). The ANG-(1–7) RIA fully recognizes ANG-(1–7) and ANG-(2–7), but cross-reacts <0.01% with ANG-(3–7), ANG II, ANG I, and their fragments. The ANG II RIA equally recognizes ANG III, ANG-(3–8), and ANG-(4–8), but cross-reacts <0.01% with ANG I and ANG-(1–7). The limit of detection was 1 fmol/tube for ANG I, 0.5 fmol/tube for ANG II, and 4 fmol/tube for ANG-(1–7) (4, 35, 36).
**125I-ANG I Metabolism**

125I-ANG I processing was measured at 37°C in the metabolism buffer using the mitochondrial lysate (50 μg) in a final volume of 250 μL. Each reaction contained a final concentration of 0.5 nM 125I-ANG I and 100 nM ANG I with or without the inhibitors N-[N-[1-(S)-carboxyl-3-phenylpropyl]-L-(S)-phenyl-alanyl]-L-isoserine (SCH; 10 μM) and SCH (10 μM)/N-[1-(R,S)-carboxyl-3-phenylpropyl]-L-alanine-Phe-p-aminobenzoate (CPP; 10 μM). The reactions were stopped after 60 min by addition of ice-cold 1.0% phosphoric acid and centrifugation at 16,000 g. The supernatants were immediately filtered, and the products were detected by HPLC-γ detection as previously described (34, 53). Preliminary studies also assessed 125I-ANG II metabolism using an identical approach as that for the mitochondrial metabolism of ANG I.

**Statistical Analysis**

All measurements are expressed as means ± SE. Differences between the groups were analyzed by one-way ANOVA and Newman-Keuls multiple comparison analysis. Statistical analyses were performed and figures constructed with GraphPad Prism V (GraphPad Software, San Diego, CA). A probability value of <0.05 was required for statistical significance.

**RESULTS**

**Angiotensinogen and Renin**

We verified mitochondrial enrichment and purity utilizing immunoactive probes against proteins to outer mitochondrial (VDAC) and inner mitochondrial (ATP5A, UCRC2, and SDHB) membranes. Immunoreactivity for VDAC (Fig. 1A), ATP5A, UCRC2, and SDHB (Fig. 1B) was enriched as mitochondria were purified through differential centrifugation and a discontinuous Percoll gradient. In contrast, expression of the lysosomal and renin granule marker cathepsin B (Fig. 1C) in addition to the cellular protein β-actin was absent in the final Percoll fraction (Fig. 1, A–C). Based on the mitochondrial purity obtained by the Percoll gradient, this preparation was utilized in subsequent experiments to characterize the RAS components.

We initially assessed renin and the prorenin receptor expression in three mitochondrial homogenates and a renal cortex homogenate. Two distinct protein bands of 37 and 55 kDa were identified utilizing both the AVIVA renin antibody (Fig. 2A) and the Inagami renin antibody (Fig. 2B). Despite the presence of renin, there was no immunological evidence of the prorenin receptor (ATP6IP2) using the Abcam antibody in three separate mitochondrial preparations, although we noted a protein band of 35 kDa in the cortical homogenate (Fig. 2C). We then assessed renin activity in isolated mitochondria from male (Fig. 3, A, C, E, and G) and female (Fig. 3, B, D, F, and H) sheep by measuring the disappearance of intact angiotensinogen from nephrectomized sheep plasma (NSP) in the absence or presence of the renin inhibitor aliskiren (Fig. 3). The renin assay was facilitated by the specificity of the ANG I-angiotensinogen antibody to recognize the ANG I sequence of angiotensinogen. As shown in the immunoblot (Fig. 3, A–D), renin activity in isolated mitochondria was evident in the time-dependent disappearance of intact angiotensinogen at 37°C and that ANG I-angiotensinogen processing was abolished by aliskiren (Fig. 3, G and H). We did not attempt to quantify renin activity between the male and female mitochondria isolated from the renal cortex. To determine prorenin levels, mitochondria were pretreated with trypsin to process prorenin to active renin, and angiotensinogen processing was determined in the presence of the trypsin inhibitor SBTI. Trypsin activation of mitochondrial prorenin did not appear to markedly increase renin activity as the disappearance rate of ANG I-angiotensinogen was comparable for the trypsin- and non-trypsin-treated mitochondria samples in males and females (Figs. 3, C and D and E and F).

The protein precursor angiotensinogen was assessed by immunoblotting using two distinct antibodies: a “total” angiotensinogen antibody that targeted an internal amino acid sequence distal to ANG I and the ANG I-angiotensinogen antibody directed against the ANG I sequence (4). Thus the ANG I-angiotensinogen antibody detects only intact ANG I-containing angiotensinogen, and the total angiotensinogen antibody detects both intact and des-ANG I angiotensinogen (i.e., renin-processed angiotensinogen). The total angiotensinogen antibody recognized a 55-kDa protein band in three different male...
mitochondrial preparations and in NSP; however, the ANG I-angiotensinogen antibody failed to detect a protein band in the mitochondrial homogenates (Fig. 4A). The absence of ANG I-angiotensinogen may suggest active processing of the precursor; therefore, we determined the expression of angiotensin peptides. Mitochondrial ANG I, ANG II, and ANG-(1–7) content was then quantified using three direct RIAs targeted to each peptide in isolated mitochondria from three separate preparations (Fig. 4B). The mitochondrial content of ANG II and ANG-(1–7) were both significantly higher than ANG I [23 ± 8 and 58 ± 17 vs. 2 ± 1 fmol/mg protein; P < 0.01, n = 3].

**ANG I Processing**

We examined the potential processing pathway of ANG I in isolated mitochondrial homogenates from sheep renal cortex using the $^{125}$I-labeled peptide as a substrate. As shown in the chromatographs for Fig. 5, $^{125}$I-ANG I was metabolized pri-

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**Fig. 3.** Renin activity in the mitochondria. Renin activity in the mitochondria of a male (A, C, E, and G) and female (B, D, F, and H) sheep. Non-trypsin (A and B)- or trypsin (C and D)-treated mitochondrial homogenates with (+) or without (−) the renin inhibitor aliskiren was detected by immunoblot analysis using an ANG I (AI)-angiotensinogen (Aogen)-directed antibody. Relative quantification of the ANG I-Aogen band without (E and F) or containing aliskiren (G and H) was performed. The disappearance or half-life ($t_{1/2}$) of AI-Aogen was determined from each experiment.

**Fig. 4.** Angiotensinogen (Aogen) and angiotensin peptide expression in isolated mitochondria from sheep renal cortex. A: immunoblot of Aogen in nephrectomized sheep plasma (NSP; lane 1) and isolated mitochondria from the renal cortex of 3 male sheep (lanes 2–4). A protein band of 55 kDa was detected for total Aogen in all lanes (top), but AI-Aogen was not detected in the mitochondria preparations (bottom). B: angiotensin peptide expression was quantified in isolated mitochondria from sheep renal cortex using distinct radioimmunoassays. ANG I, ANG II, and ANG-(1–7) were detected; however, there was a significant difference between the levels of ANG I compared with ANG II and ANG-(1–7). Values are means ± SE; n = 3. *P < 0.01 ANG I vs. ANG II, ANG I vs. ANG-(1–7).
arily to $^{125}$I-ANG-(1–7). Addition of the neprilysin inhibitor SCH reduced the $^{125}$I-ANG-(1–7) peak (Fig. 5B). The coaddition of SCH and the thimet oligopeptidase inhibitor CPP further reduced the $^{125}$I-ANG-(1–7) peak and preserved the $^{125}$I-ANG I peak (Fig. 5C). Immunoblot analysis of the 100,000-g mitochondrial membranes revealed NEP expression while the corresponding 100,000-g soluble fraction expressed thimet oligopeptidase (TOP) in mitochondrial homogenates (lanes 2–4, n = 3) along with renal cortex homogenate as a positive control (lane 1, D). $^{125}$I-labeled products were separated by HPLC under gradient conditions. Statistical quantification of $^{125}$I-ANG I metabolism demonstrated SCH (10 µM) and SCH in combination with CPP (10 µM) reduced $^{125}$I-ANG-(1–7) formation (E and F). The addition of SCH and CPP did not significantly further influence $^{125}$I-ANG-(1–7) formation or preservation of $^{125}$I-ANG I (E and F). Values are means ± SE; n = 3. *P < 0.05 vs. control (CON).

**DISCUSSION**

Compelling evidence strongly supports an intracellularly based RAS in the kidney that contributes to renal function, as well as the regulation of oxidative stress (3, 25, 26, 29, 42). Mitochondria are now considered a key organelle regarding their role in chronic cardiovascular diseases and the mechanisms that underlie hypertension and renal dysfunction (10, 14, 16, 38, 45). Mitochondria are one of several intracellular organelles that contain elements of a RAS, and ANG II may directly influence oxidative stress and mitochondrial respiration (1, 5, 21). The present study provides further support for an intramitochondrial RAS pathway that is distinct from the peripheral RAS. Although previous reports have indicated associations between RAS components and mitochondrial function (1, 5, 21, 45, 47), the biochemical approach in this study allowed for a more complete characterization of a mito-

Expression of the Mas Receptor

In lieu of the mitochondrial content of ANG-(1–7), we further assessed whether these organelles express the protein for the ANG-(1–7)/Mas receptor. For these studies, we utilized the Alomone antibody against the Mas receptor. We previously demonstrated that the antibody identified the receptor protein within the cortical tubules of the sheep kidney by immunofluorescent staining (26). Using a new batch of the antibody, the immunoblot revealed a single band of 40 kDa in the cortical homogenate, crude mitochondrial pellet, and the purified Percoll fraction from the sheep renal cortex (Fig. 6A). A single immunoreactive band for the Mas protein was also evident in the Percoll mitochondrial fraction from three male sheep (Fig. 6B).

![Fig. 5. Mitochondrial processing of $^{125}$I-ANG I. Chromatograph reveals $^{125}$I-ANG I ($^{125}$I-AI) was metabolized to $^{125}$I-ANG-(1–7) ($^{125}$I-A7) in renal mitochondrial homogenate fractions (A). $^{125}$I-AI metabolism to $^{125}$I-A7 was reduced by 10 µM SCH (B), and $^{125}$I-AI metabolism to $^{125}$I-A7 was reduced by 10 µM SCH in combination with 10 µM CPP (C). Immunobots for neprilysin (NEP) and thimet oligopeptidase (TOP) in mitochondrial homogenates (lanes 2–4, n = 3) along with renal cortex homogenate as a positive control (lane 1; D). $^{125}$I-labeled products were separated by HPLC under gradient conditions. Statistical quantification of $^{125}$I-ANG I metabolism demonstrated SCH (10 µM) and SCH in combination with CPP (10 µM) preserved $^{125}$I-ANG I, while SCH (10 µM) and CPP (10 µM) reduced $^{125}$I-ANG-(1–7) formation (E and F). The addition of SCH and CPP did not significantly further influence $^{125}$I-ANG-(1–7) formation or preservation of $^{125}$I-ANG I (E and F). Values are means ± SE; n = 3. *P < 0.05 vs. control (CON).
Fig. 6. Immunodetection of receptor expression in sheep renal mitochondria. A: whole renal cortex homogenate (lane 2), crude mitochondrial fraction (lane 3), and Percoll-purified mitochondrial fraction (lane 4) were subjected to 10% SDS gel and immunoblotting with an antibody against the MAS receptor protein. B: whole renal cortex homogenate (lane 2) and purified mitochondria (lane 3–5) from 3 distinct male sheep exhibited protein expression of the MAS receptor. Lane 1 (A and B) is the protein molecular weight marker.

Mitochondrial RAS regarding the alternative ANG-(1–7) axis. Our studies revealed that mitochondrial renin was active and cleaved exogenous angiotensinogen. Mitochondria expressed the des-ANG I form of angiotensinogen, but not intact angiotensinogen, suggesting that renin may process endogenous angiotensinogen within the mitochondria. We also show by direct RIA the presence of the angiotensin peptides ANG I, ANG II, and ANG-(1–7) within mitochondria. Moreover, the mitochondrial preparation processed ANG I to ANG-(1–7) by the endopeptidases neprilysin and thimet oligopeptidase. Finally, the current study revealed that purified mitochondria expressed a single protein band for the AT7/Mas receptor.

Peters and colleagues (44) originally identified an alternative transcript of renin that lacked exon 1 which precluded entry of the transcribed protein into the secretory pathway; this isoform was predicted to reside within the cell. These investigators subsequently showed that the truncated form of renin was catalytically active and preferentially localized to the mitochondria (9). Overexpression of this renin isoform in a human cardiomyocyte cell line was recently shown to protect the cells from necrosis in response to reduced glucose conditions; this protective effect was not reversed by a renin inhibitor, suggesting a direct effect of renin rather than renin-dependent processing of angiotensinogen (52). Clausmeyer et al. (9) reported that truncated renin was actively taken up and internalized by isolated mitochondria, but that the full-length prorenin form did not undergo internalization. Ishigami et al. (27) have recently demonstrated an additional renin transcript that lacks a portion of the pre-pro segment such that the enzyme is constitutively active; the isoform constitutes ~10% to total renin and was expressed predominantly along the tubular elements of the mouse kidney. The forced overexpression of this renin isoform was associated with higher blood pressure in the transgenic mice (27). Although the Ishigami study found no change in circulating renin suggesting that the renin isoform is not secreted, the intracellular localization of the renin isoform within the tubules was not established (27). It is not currently known whether different renin transcripts are expressed within the sheep kidney or the extent to which renin isoforms traffic to the mitochondria.

The renal cortex consists of multiple cell populations, and although tubules constitute the major cell type, we cannot definitively exclude juxtaglomerular or other cell contamination. Juxtaglomerular cells are the major source of renin and contain both prorenin and active forms of renin (28, 37). Renin-containing granules sediment at a higher density than purified mitochondria; however, the possibility of renin contamination from granules is plausible, and isolated mitochondria were probed with a cathepsin B antibody. Renin and cathepsin B coexist within renin granules, and cathepsin B is a major component of lysosomes (50). Both cathepsin B and β-actin protein expression were diminished as mitochondria were purified from sheep renal cortex, suggesting a negligible contamination of renin granules or lysosomes in the mitochondrial preparation. Moreover, trypsin treatment did not result in a marked increase in renin activity. The inability of trypsin to enhance renin activity further argues for the lack of renin granule contamination in the isolated mitochondria. We found no immunological evidence for the prorenin receptor in mitochondria, which is consistent with predominant intracellular localization of this receptor on the endoplasmic reticulum, as well as the plasma membrane (4, 26, 27, 52).

Evidence of catalytically active renin in the mitochondria would support the predominant des-ANG I form of angiotensinogen in this organelle. Peters et al. (44) originally identified angiotensinogen by electron microscopy (EM) in mitochondria from the adrenal cortex along with other RAS components. Moreover, our preliminary data suggest that angiotensinogen is internalized by isolated mitochondria from both the sheep renal cortex and human HK-2 proximal tubule cells (54). Evidence for the angiotensin peptides ANG I and ANG-(1–7) within the mitochondria may also reflect the renin-dependent processing of angiotensinogen. ANG I metabolism studies revealed the generation of ANG-(1–7) that was attributed to the endopeptidases neprilysin (EC 3.4.24.11) and thimet oligopeptidase (EC 3.4.24.15). Neprilysin contributes to the generation of circulating ANG-(1–7), particularly following ACE inhibition; however, to our knowledge, these are the first studies to indicate neprilysin activity within the renal mitochondria (4, 19, 40, 53). Although the current understanding of a mitochondrial role for neprilysin is limited, there is evidence for the peptidase in mitochondrial-mediated amyloid β degradation (33). Transgenic mice engineered to produce amyloid β that contained the human mitochondria-targeted antioxidant catalase were found to upregulate neprilysin expression that was associated with reduced amyloid β and oxidative DNA damage (33). A role for thimet oligopeptidase in the intracellular processing of ANG I to ANG-(1–7) was evident in rat vascular smooth muscle cells, the NRK-52E renal epithelial cell line, and the rat brain (4, 7, 43). The enzyme is primarily an intracellular metallopeptidase of the M3 family of metallopeptidases and hydrolyzes peptides with an optimal length of 9–17 residues.
The biochemical detection of ANG II corroborates several studies that have localized ANG II to the mitochondrial by immunogold EM (1, 18, 44). We did not detect ACE activity in isolated mitochondria from the renal cortex, consistent with the findings of Astin et al. (5) in liver mitochondria. Moreover, we found no evidence of other ANG II-forming activities (cathepsin G, elastase-2, chymase) from ANG I in the absence or presence of the combined neprilysin and thimet oligopeptidase inhibitors that abolished ANG-(1–7) formation, and these data do not support the conversion of ANG I to ANG II within mitochondria (8). However, Peters and colleagues (44) detected immunoreactive ACE in mitochondria isolated from the rat adrenal cortex, and it is possible that species or tissue differences may underlie apparent discrepancies in the expression of RAS components in this organelle. Alternatively, both the Abadir (1) and Astin (5) studies reported AT1 receptors in isolated mitochondria and mitochondrial ANG II may derive from trafficking of the ANG II-AT1 receptor complex from the cell surface. Early studies by Sirett et al. (48) described the highest density of ANG II binding sites on the purified mitochondrial fraction from the rat brain. Moreover, Goodfriend and colleagues (21) originally reported direct effects of ANG II on mitochondrial phosphorylation in isolated preparations. Danser and colleagues (51) found that uptake of circulating 125I-ANG II by the kidney was also associated with the mitochondrial fraction, and Li et al. (30) recently demonstrated the trafficking of the ANG II-AT1 receptor complex to the mitochondria in proximal tubule cells. We also identified the AT1/Mas receptor on purified mitochondria based on Western blot analysis using the Alomone antibody and had previously demonstrated Mas expression primarily on the tubular elements of the sheep kidney with the same antibody (26). AT1, AT2, and Mas receptors do not express a canonical N-terminal mitochondrial targeting sequence, nor are these receptors predicted to localize to the mitochondria based on subcellular localization prediction software (TargetP 1.1 Server) (17). Moreover, to our knowledge, there is no evidence that mammalian mitochondrial DNA genes code for these receptors based on the MitoCarta inventory (6). However, AT1, AT2, and Mas receptor subtypes are evident on other intracellular organelles, including the nucleus and endoplasmic reticulum; thus there may exist alternative mechanisms for the intracellular expression of angiotensin receptors (1, 4, 22–24, 26, 31, 32, 41, 42, 47, 49).

The present study isolated mitochondria from the renal cortex, and although the tubular epithelial cells are the most predominant cell type in this tissue distinguished by a high density of mitochondria, we cannot definitely localize all the RAS elements to one particular cell type at this time. However, it should be noted that Peters et al. (44) localized multiple components of the RAS, including immunoreactive renin, angiotensinogen, ACE, and ANG II, to the mitochondria of the rat adrenal cortex. Moreover, we did not evaluate any functional properties of ANG-(1–7) or ANG II within the sheep mitochondria. The ANG-(1–7)-AT1/Mas receptor axis may act as a physiological antagonist of ANG II that counterbalances the deleterious effects of ANG II-mediated intracellular signaling (7, 38). In this regard, ANG-(1–7) attenuated mitochondrial ROS production and cellular apoptosis induced by ANG II in renal NRK-52E epithelial cells (29). Since the ANG-(1–7)-AT1/Mas receptor axis is typically linked to NO generation in the kidney and other tissues, investigation of this pathway to influence mitochondrial function under normal and pathological conditions may be warranted, particularly as ANG-(1–7) was recently shown to be more effective than the AT1 receptor antagonist to attenuate renal injury in a model of diabetic nephropathy (55).

**Perspectives and Significance**

The RAS may influence kidney dysfunction and oxidative stress maintenance within a variety of intracellular organelles (2). In general, the ANG-(1–7)-AT1/Mas axis opposes or functionally antagonizes a stimulated ACE-ANG II-AT1R pathway. The renal actions of an intramitochondrial ANG-(1–7) system may encompass the release of NO, activation of antiapoptotic pathways, and/or the reduction of oxidative stress. Moreover, the loss of ANG-(1–7) tone within the kidney may accelerate deleterious mitochondrial pathways that increase oxidative stress and enhance apoptosis under pathological conditions. Therefore, the presence of a mitochondrial ANG-(1–7)-AT7/Mas axis within the kidney could constitute a novel therapeutic target to influence intracellular levels of oxidative stress and respiration; elucidation of the peptide’s functional role may identify specific intracellular targets to attenuate renal disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.A.W. performed experiments; B.A.W. analyzed data; B.A.W. and M.C.C. interpreted results of experiments; B.A.W. prepared figures; B.A.W. and M.C.C. drafted manuscript; B.A.W., M.N., T.M.G., J.C.R., and M.C.C. edited and revised manuscript; B.A.W., M.N., T.M.G., J.C.R., and M.C.C. approved final version of manuscript; M.C.C. provided conception and design of research.

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