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No evidence for a local renin-angiotensin system in liver mitochondria

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The circulating, endocrine renin-angiotensin system (RAS) is important to circulatory homeostasis, while ubiquitous tissue and cellular RAS play diverse roles, including metabolic regulation. Indeed, inhibition of RAS is associated with improved cellular oxidative capacity. Recently it has been suggested that an intra-mitochondrial RAS directly impacts on metabolism. Here we sought to rigorously explore this hypothesis. Radiolabelled ligand-binding and unbiased proteomic approaches were applied to purified mitochondrial sub-fractions from rat liver, and the impact of AngII on mitochondrial function assessed. Whilst high-affinity AngII binding sites were found in the mitochondria-associated membrane (MAM) fraction, no RAS components could be detected in purified mitochondria. Moreover, AngII had no effect on the function of isolated mitochondria at physiologically relevant concentrations. We thus found no evidence of endogenous mitochondrial AngII production, and conclude that the effects of AngII on cellular energy metabolism are not mediated through its direct binding to mitochondrial targets.

The circulating (endocrine) renin-angiotensin system (RAS) plays a key role in human circulatory homeostasis. Hepatically-derived angiotensinogen is cleaved by the aspartyl protease renin of renal juxtaglomerular origin to yield the inert decapeptide angiotensin I (AngI). Circulating or endothelially-bound angiotensin-I converting enzyme (ACE) converts AngI to octapeptide angiotensin II (AngII), which promotes renal salt and water retention (through aldosterone released from the adrenal gland), whilst also causing arteriolar vasoconstriction. In these ways, the endocrine RAS promotes intravascular fluid retention and help maintain arterial blood pressure¹. Meanwhile, ubiquitous local tissue RAS synthesise AngII which acts on adjacent cells (paracrine actions), on the surface of the synthesizing cell itself (autocrine actions), or on intracellular receptors, often found in the nucleus (intracrine actions). Such local RAS may be complete, or dependent for their function on the uptake of some critical RAS components from the circulation, with some cells internalising exogenous AngII, and others synthesising it de novo²⁻⁵. Whether of local or systemic origin, AngII mediates its effects through action at two receptor subtypes. While the role of its type-2 receptor (AT₂R) is less clear, the type-1 receptor (AT₁R) mediates diverse responses, amongst them the regulation of inflammation, fibrosis, cell growth and survival^{6,7}.

Recent studies suggest that AngII may also play an important role in the regulation of cellular energy metabolism. In humans, genetically-determined lower ACE activity is associated with enhanced efficiency, reduced oxygen consumption per unit of external work and a relative conservation of fat stores during exercise, as well as with increased performance in hypoxic environments⁸⁻¹². In rodents, combined ACE inhibition and AT₁R antagonism reduce renal oxygen consumption related to sodium transport¹³, while infusion of AngII increases oxygen consumption in different tissues^{14,15}. In addition, AngII has been shown to modulate mitochondrial membrane potential, expression of uncoupling proteins and transcription of respiratory chain subunits, and to trigger the generation of reactive oxygen species (ROS)¹⁶⁻¹⁸.



Mitochondrial effects of AngII might be mediated by activation of cellular signalling pathways through AngII action on cell surface receptors^{6,19}. Alternatively, AngII may have direct effects upon mitochondria, given that AngII and AT₁R have been observed on the outer mitochondrial membrane (OMM)^{20,21}, and that exogenously-administered ³H-labelled AngII has been shown to traffic to the surface of rodent mitochondria²². In addition, however, it has also been suggested that a bona fide intra-mitochondrial RAS might exist, capable of de novo AngII synthesis. Interest in the existence of such a system has increased by a recent report which suggested the presence of AT₂R on the inner mitochondrial membrane²³. However, this conclusion was largely based upon the use of AT₂R antibodies whose specificity was untested in this context, and on non-quantitative imaging.

We thus sought to further explore the presence of a mitochondrial RAS through the application of unbiased proteomic approaches and radiolabelled ligand binding in highly purified mitochondrial fractions from rat liver, together with mitochondrial functional assays. Our results exclude the presence of intramitochondrial AT receptors and other components of RAS, but show that AT₁R are present in the MAM. Specific binding of AngII to these receptors did not elicit physiological effects on mitochondrial respiration in isolated liver mitochondria, contesting the generalised relevance of direct mitochondrial actions of RAS.

Results

Mass spectrometry and in silico analysis of the mitochondrial proteome do not verify the existence of a mitochondrial RAS.

First, in order to obtain unbiased evidence for the presence of RAS in mitochondria, we used purified mitochondrial fractions for proteomic analysis. The Crude mitochondrial fraction (CM), along with nuclei, microsomes, lysosomes and cytoplasm were purified by differential centrifugation. From the CM fraction, pure mitochondria (PM) were separated from mitochondria associated membranes (MAM), using isopicnic ultracentrifugation on a self-forming Percoll density gradient from rat livers²⁴. The MAM fraction represents the interface of mitochondria with other cellular organelles, in particular the endoplasmic reticulum (ER), where signalling and metabolic interactions take place. It thus contains components of the OMM and other loosely associated cellular membranes. In contrast, pure mitochondria are devoid of other organelles, and highly enriched in matrix, IMM, OMM and intermembrane space components (for recent reviews see^{25,26}). Proteins from the CM, PM and MAM fractions were separated by SDS-PAGE and subjected to mass spectrometry analysis (Supplementary Fig. S1 and Supplementary Dataset S1). We compiled a list of RAS-related genes using the AmiGO gene ontology (GO) database, and sought the presence of their transcription products amongst those identified by mass spectrometry analysis. Importantly, from the three RAS related components found in the CM and MAM and PM fractions, none is involved directly in angiotensin generation and binding (see Supplementary Dataset S1).

The validity of these findings is supported by interrogation of unbiased catalogues of the mitochondrial proteome. The MitoMiner database aggregates findings from 47 proteomic surveys across several species²⁷, while MitoCarta combines proteomics, imaging and sequence analysis to score the probability of mitochondrial localization of individual proteins in humans and mouse, further increasing the sensitivity to identify mitochondrial proteins²⁸. In addition to our proteomic analysis in rat liver, the use of these databases allowed us to test the presence of RAS components and all related genes from a series of mammalian species and tissues, including rodent, bovine and human gene sets (Supplementary Dataset S2). Again, we generated gene sets from those belonging to all RAS related GO terms in the AmiGO database across all species. We then searched against the predicted mitochondrial genes in the MitoMiner and MitoCarta

databases. This revealed mitochondrial localization of gene products involved in aldosterone synthesis, as targets of RAS, but no intrinsic RAS components have been experimentally proven or were bioinformatically predicted to localize to mitochondria (Supplementary Dataset S2).

While these approaches together rendered the presence of RAS in the mitochondria unlikely, they cannot formally exclude the possibility of the presence of components at low abundance. Thus, we proceeded to analyse the purified mitochondrial sub-fractions for the presence of main RAS components using immunoprecipitation and immunoblotting.

Rat liver mitochondria do not contain detectable ACE. If a functional, self-sufficient RAS exists in mitochondria, it should contain enzymes generating AngII, a role mostly fulfilled by ACE, the most evolutionarily conserved enzyme, which is directly responsible for the generation of AngII from AngI throughout the body. We thus sought to identify ACE in purified mitochondria from rat liver tissue. As shown in Fig. 1A, an antibody directed to the C-terminus of the protein recognised a high molecular weight band (MW ≈ 150 kDa, predicted MW of ACE) in the homogenate, nuclear, lysosomal and microsomal fractions, but not in mitochondria. Conversely, a low MW (≈50 kDa) band was enriched in the crude mitochondrial fraction. This band was also present in the pure mitochondrial fractions from three independent preparations albeit at lower and variable intensity. Since a shorter natural human and rat ACE isoform exists (ACE-T, Uniprot P47820), we sought to confirm the identity of the lower MW mitochondrial band. We thus purified it by immunoprecipitation (Fig. 1B) and analysed by mass spectrometry. However, the results of the analysis did not return any ACE related sequences, indicating that the band represents non-specific binding by the antibody. Altogether, these results confirmed the proteomic analysis excluding the presence of ACE in mitochondria.

AT₁R is present in the MAM, but no AT₁R, AT₂R or AngII binding is detectable in the PM fraction. Whilst the previous results excluded the possibility of intra-mitochondrial generation of AngII by ACE, mitochondria can still be the target of AngII generated at other intracellular sites or imported from extracellular sources. We therefore sought the presence of functional AngII receptors in rat liver subcellular and submitochondrial fractions, using two independent strategies. First, we measured specific binding of [¹²⁵I]-AngII in the PM and MAM fractions. As shown in Fig. 2A, we detected a single high affinity binding site in the MAM fraction as indicated by Hill slopes close to unity, with a subnanomolar equilibrium dissociation constant (K_D) and a maximal receptor density (B_{max}) comparable to the range found in the plasma membrane²⁹, indicating that functional AngII binding sites are present in mitochondria associated membranes. However, the same high affinity binding sites showed ≈ 50 times less density in purified mitochondria, most likely indicating contamination from the MAM fraction, and unlikely to be consistent with efficient AngII mediated signalling. Binding of AngII to the MAM-localized binding sites was inhibited by the specific AT₁R antagonist Losartan, but not affected by the AT₂R antagonist PD123319, indicating that binding was AT₁R-related (Fig. 2B).

In order to further analyse the presence and localization of AT₁R and AT₂R subtypes, we next performed Western blot analysis of the rat liver subcellular and sub-mitochondrial fractions. In agreement with the binding studies, we were able to detect AT₁R in the CM and MAM fraction, but not in the PM (Fig. 2C). In contrast, antibodies against AT₂R gave a series of immunoreactive bands (Fig. 3A), even in the expected molecular weight range of the receptor (≈40–50 kD; Fig. 3B). Thus we followed the strategy previously applied to ACE, using the antibody to immunoprecipitate its binding partners and identified the resulting proteins by mass spectrometry. Immunoprecipitation with the anti-AT₂R antibody pulled down three bands in the range of 30–70 kD in the MAM and partly in the PM fraction

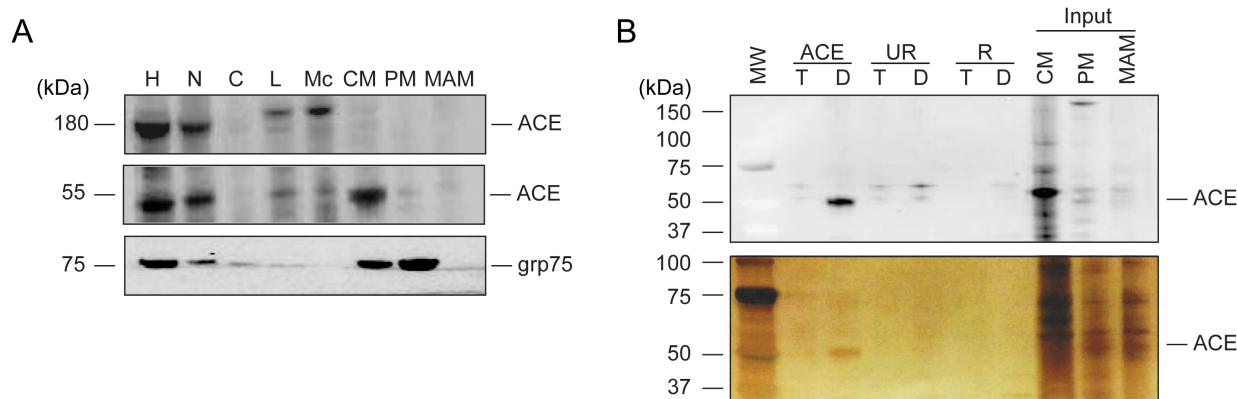


Figure 1 | Mitochondrial subfractions lack detectable ACE. (A). Western blot using anti ACE and anti-grp75 antibodies from rat liver subcellular fractions. H-homogenate, N-nuclear fraction, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation to obtain crude mitochondria (CM) and further separated to mitochondria associated membranes (MAM) and pure mitochondrial (PM) fractions by isopicnic Percoll centrifugation (for details see Methods). Mitochondrial fractions contain only a non-canonical 50 kDa immunoreactive band. Images of immunoblots were cropped to delineate the regions of interest. For the two immunoblots different aliquots of the same samples were loaded and separated under identical conditions. See full images on Supplementary Figure S3. (B). Immunoprecipitation of ACE using the CM fraction as input using the Pierce Crosslink IP approach as described in Methods. 10 μ g ACE C-20 antibody was crosslinked to the protein A/G agarose resin and 1 mg protein was used as input. The immunoprecipitation was performed either in Tris-Buffered Saline, (TBS, 0.025 M Tris, 0.15 M NaCl; pH 7.2) or TBS supplemented with 0.5 mM EDTA, 0.5% NP-40, 2.5% glycerol to promote ACE binding (D). An unrelated goat antibody (UR) and the resin without antibody crosslinked (R) were used as controls in both TBS and D solutions. Upper panel shows immunodetection using the same ACE antibody, lower panel shows silver stained SDS-PA gels. The 50 kDa band in the ACE-D fraction has been analysed by mass spectrometry, and identified as a non-ACE or RAS related protein. Images were cropped to show all visible bands.

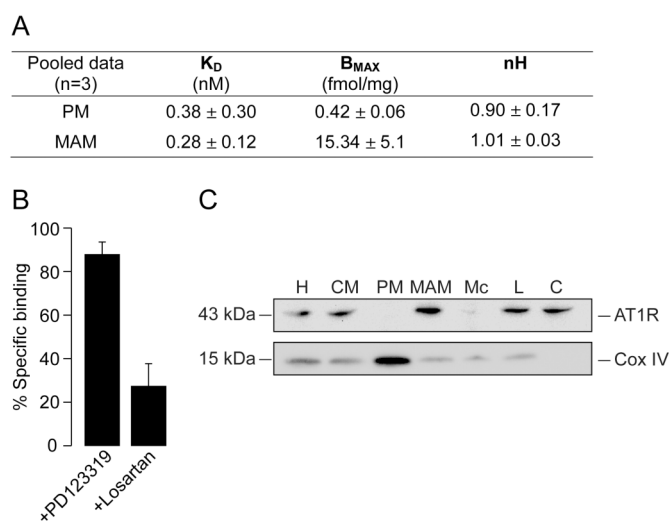


Figure 2 | AT₁R is present in the MAM fraction. (A). Affinity (K_D), density (B_{MAX}) and cooperativity (Hill coefficient, nH) of specific AngII binding sites in the PM and MAM fractions. (B). Specific binding of [¹²⁵I]-AngII in the MAM fraction in the presence of AT₁R (losartan) or AT₂R (PD123319) blockers. The concentration of losartan was calculated from inhibition constants²⁹ to block >95% of the AT₁ receptor but <2% of AT₂R. [PD123319] was calculated to block 99% of AT₂R but <2% of AT₁R. (C). Western blot detection of AT₁R in subcellular fractions. H-homogenate, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation (CM) and further separated to mitochondria associated membranes (MAM) and pure mitochondrial (PM) fractions by isopicnic Percoll centrifugation (for details see Methods). Cytochrome oxidase subunit IV (CoxIV) was used as mitochondrial inner membrane marker. Images of immunoblots were cropped to delineate the regions of interest. The same membrane was used for both immunoblots. See full images on Supplementary Figure S4.

(Fig. 3C). However, mass spectrometry identified these bands as unrelated proteins.

Together, these results confirm the lack of functional AngII receptors in rat liver mitochondria, but raised the possibility that intracellular AngII might alter mitochondrial function through agonist action at receptors located on the MAM. We thus performed further experiments using purified crude mitochondria (which contain pure mitochondria and their associated membranes) and evaluated the effect of AngII on oxidative phosphorylation.

AngII exerts marginal inhibition on respiration of isolated mitochondria at supra-physiological concentrations. Physiological concentrations of AngII in the plasma are in the picomolar range, in accordance with the sub-nanomolar affinity of angiotensin receptors on the cell surface. We have found AT₁Rs with similarly high affinity in the MAM fraction. Tissue AngII levels vary from picomolar to the low nanomolar range depending on tissue type and conditions^{30,31}, while no intracellular concentrations of AngII have been reported so far. Thus, we analysed the effect of AngII on isolated mitochondria (crude mitochondrial fraction, containing both the PM and MAM sub-fractions) in a broad concentration range. Supra-physiological (1 μ M) saturating AngII had no significant effect on basal endogenous mitochondrial respiration rate (5 to 25 min in the absence of exogenous substrates and ADP) as compared to controls (Figure 4A and Supplementary Figure S2). Similarly, acute addition of AngII, using concentrations in the physiological range (10–100 nM) had no effect on the activity of complexes I and II in the presence of substrates and ADP (state 3 respiration, Fig. 4B–D). Finally, we applied AngII in the range of 1 nM–1 μ M range for 20 min and measured the state 3 activities of complexes I, II and IV (Fig. 4E–G). Again, AngII exerted no significant effect on respiration when applied in the physiological range (1–100 nM), while 1 μ M AngII induced a minor but significant reduction in the maximal ADP-stimulated complex I-, II, and IV-dependent state 3 respiration. These results suggest that AngII does not exert a specific effect on oxidative phosphorylation in mitochondria isolated from rat liver, but might target non-specific binding sites at supra-physiological concentrations.

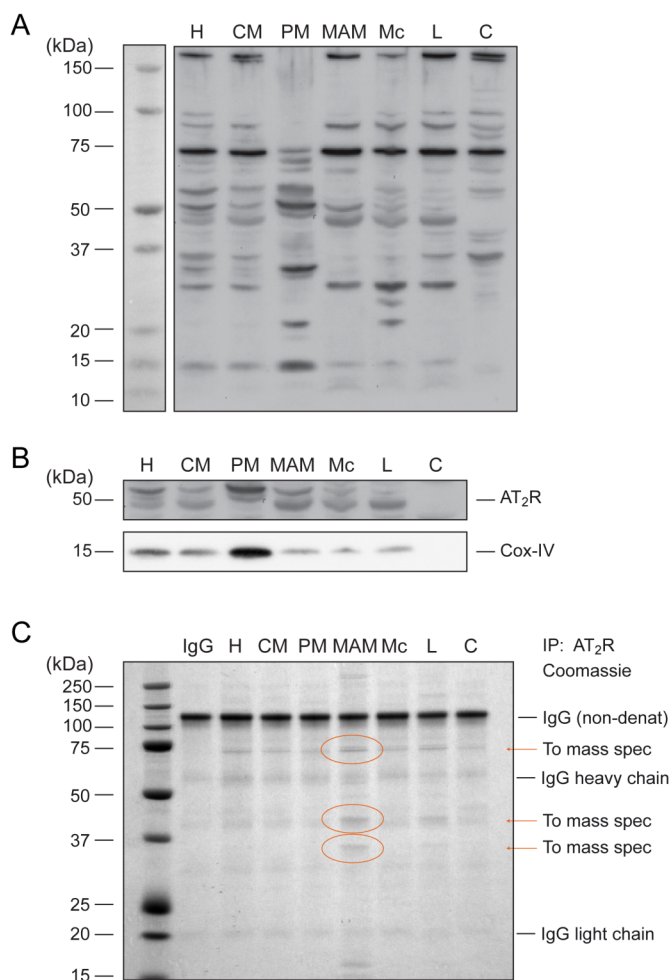


Figure 3 | Lack of an AT₂R specific immunoreactive band in rat liver mitochondria. (A). Immunoreactive bands detected by the sc9040, rabbit AT₂R antibody in subcellular fractions: H-homogenate, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation (CM) and further separated to mitochondria associated membranes (MAM) and pure mitochondrial (PM) fractions by isopycnic Percoll centrifugation (for details see Methods). For immunoblotting the same membrane was used as in Fig. 2C. Images were cropped to show all visible bands. The bands appearing ~ 15 kD represent CoxIV staining. (B). The method reveals several bands also in around the predicted molecular weight of AT₂R. The AT₂R panel was cropped from (A). Cytochrome oxidase subunit IV (CoxIV) was used as mitochondrial inner membrane marker, immunoblotted from the same membrane as used in (A) and Fig. 2C. The full images are shown in Supplementary Fig. S4. (C). AT₂R immunoprecipitations from all subcellular fractions and IgG controls separated by SDS PAGE and stained with Coomassie-blue. To maintain integrity of IgG at 130 kDa, we used a non-denaturing 4× loading buffer without dithiothreitol and β-mercaptoethanol (200 nM Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.1% bromophenol blue). Three immunoprecipitated bands from the MAM fraction (red circles) have been identified by mass spectrometry as non-RAS related proteins. Images were cropped to show all bands.

Discussion

In view of the accumulating evidence of metabolic effects of the circulating and local RAS in mammals, it is an attractive hypothesis that intracrine RAS evolved to directly influence mitochondrial function. To test this assumption we have considered two scenarios.

First, previous work suggested that mitochondria might contain a fully functional RAS leading to intra-mitochondrial generation of

AngII. A truncated prorenin, generated by alternative splicing, was shown to contain mitochondrial targeting sequences and to be *de facto* imported into isolated mitochondria³². Moreover, intra-mitochondrial ‘dense bodies’ were shown to contain renin using immunogold labelling and electron microscopy (EM)³³, together with angiotensinogen and AngII³⁴. However, the presence of these RAS components appears to be limited to certain tissues and conditions, particularly to the adrenal cortex following nephrectomy (for a review see ref. 35). Such an intracellular RAS system has been proposed to serve as a local amplifier of RAS signalling, reducing the requirement for circulating AngII, and stimulating aldosterone secretion³⁶. Indeed, renin co-localizes with several steroid synthesis enzymes in the mitochondria, but the exact function of mitochondrial RAS components in this tissue has not yet been clarified. In addition, a study using Percoll gradient purification of crude mitochondria localised ‘dense bodies’ with functional renin activity in a mitochondria associated membrane fraction³⁷, and close inspection of EM images from the previous references also show scattered distribution of immunogold labelling around mitochondria. Thus, to clarify whether RAS is a general component of the mitochondrial proteome, we have carried out unbiased proteomic and *in silico* analyses of all known RAS components and functionally related proteins. In addition, we particularly chose to evaluate the presence or absence of ACE in mitochondria for two reasons: (i) ACE represents the final essential step in AngII generation and (ii) ACE orthologs have been detected in several bacterial species, making it the most likely candidate to be present in the ancestors of mitochondria according to the endosymbiotic theory, in contrast to the rest of the RAS components, which appeared rather late in vertebrate evolution, in parallel with the development of the juxtaglomerular apparatus in Osteichthyes (e.g. zebrafish)³⁸. Our results, including mass spectrometry, bioinformatics and immunoprecipitation/Western blot analyses, provided no support at all to the idea of a full-blown RAS operating in the mitochondrion of liver cells. Since dual, cytoplasmic/nuclear vs. mitochondrial localisation of a surprisingly large fraction of the proteome has been recently predicted³⁹, we cannot exclude that under certain conditions and in specific tissues RAS components might be imported into the mitochondrion, but the functional consequences of such an event remains obscure.

The second scenario is based on the currently widely held idea that intracrine signalling networks, including a range of peptide hormones, growth factors and enzymes (for a detailed list see e.g. a recent review by Re & Cook¹⁸), are targeting the nucleus and mitochondria to alter cellular function. In the case of AngII, this can occur by activation of nuclear receptors: nuclear targeting of AngII was already reported in the early 1970s²², and later confirmed by several groups in a range of tissues, along with AT₁Rs^{20,21,40}. Whilst the functional role of the nuclear binding of still remains still to be clarified, a number of recent observations suggest that AngII alters mitochondrial function. It is thus important to localise the target of AngII and to understand its mechanism of action. The best characterised pathway of AngII action on mitochondria via plasma membrane AT₁R receptors is linked to intracellular Ca²⁺ mobilisation following Ca²⁺ release from endoplasmic reticulum Ca²⁺ stores¹⁹. The ensuing accumulation of Ca²⁺ in mitochondria can exert acute activation of the Krebs cycle and OXPHOS⁴¹, but chronic activation of the pathway may lead to mitochondrial Ca²⁺ overload, depolarisation and reduced ATP production^{42,43}. Parallel to Ca²⁺ signalling, multiple pathways are activated by both AT₁R and AT₂R, including MAPK and reactive oxygen species (ROS) generation, which can contribute to AngII mediated mitochondrial dysfunction^{6,44}. Finally, however, intracellularly-generated or imported AngII might directly act on mitochondria. Thus, in addition to detailed proteomic analysis, we tested this hypothesis by probing direct binding of AngII to mitochondrial sub-fractions and evaluating the functional effect of AngII on isolated liver mitochondria. Our results argue against a

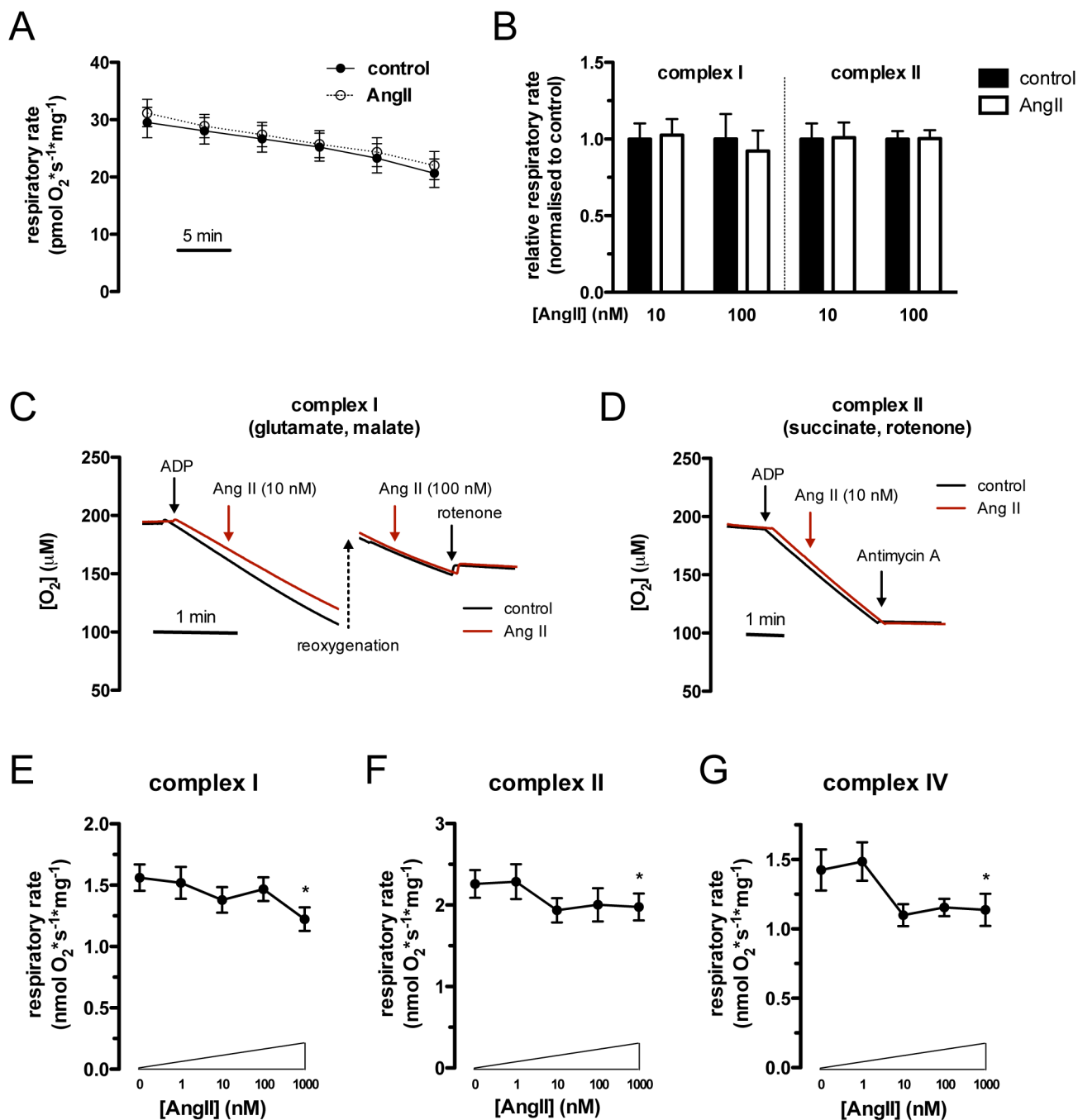


Figure 4 | The effect of AngII on respiration of isolated liver mitochondria. (A). Basal oxygen consumption of isolated rat liver mitochondrial 5 min after transfer to the respiration chamber, and at 5, 10, 15, 20 and 25 minutes after addition of AngII (1 μ M) ($n = 18$). (B–D). ADP-induced oxygen consumption of isolated rat liver mitochondria for complexes I and II, with and without (control) acute addition of AngII at the indicated concentrations. Data are summarized on panel (B). (C) and (D) show individual representative traces from 9 experiments from 3 independent preparations. (E–G). ADP-induced oxygen consumption of isolated rat liver mitochondria for complexes I, II and IV after 20 minutes of incubation with and without (control) AngII for 20 min ($n = 9$ (1–100 nM); $n = 16$ (1 μ M)). Data represent mean \pm SEM. Statistical analysis: for basal respiration rates, differences along time between samples were assessed by ANOVA for repeated measures (time-group interaction: $p > 0.05$). For ADP-stimulated respiration rates differences between samples were assessed using paired sample t-test ($* p < 0.05$ AngII vs controls for comparisons of complexes I, II and IV).

generalised view of direct action of AngII on the organelle: pure mitochondria did not significantly express specific AngII binding sites, while only marginal inhibition of OXPHOS could be observed, and this at very high [AngII].

Interestingly, AT₁Rs could be found at high density in the MAM fraction, but their origin and functional relevance is uncertain. It is possible that they represent internalised plasma membrane receptors transiently associated with membranes co-purifying with mitochondria

during intracellular trafficking. Certainly they appear dissociated from downstream signalling with functional relevance to the mitochondrion, since AngII was unable to alter mitochondrial function in our studies of the CM fraction.

Our findings are somewhat in conflict with a recent study arguing for the presence of functional AT₂Rs on mitochondria from rat heart and liver²³. We have extensively tested the specificity of the antibody used in their study. As shown on Fig. 2, the antibody labels several



bands at various molecular weights, and immunoprecipitates several unrelated proteins from liver mitochondrial fractions, as identified by mass spectrometry. The mitochondrial immunogold and immunofluorescence labelling results reported by Abadir et al. should thus be interpreted with significant caution. Moreover, the study applied only CGP 42112, an AT₂R agonist, which has been reported to antagonise the AT₁R as well⁴⁵, and functional impacts of natural AT receptor ligands were not demonstrated.

Altogether, our results rule out the generalised presence of RAS in mitochondria as well as the direct action of intracrine AngII on the organelle. Further studies of other tissues, and of human origin, are required in order to assess mitochondria as specific targets of pharmacological interventions aimed at modifying the pathological effects of RAS.

Methods

Reagents and antibodies. AngII was purchased from Alexis Biochemicals (UK and Switzerland). [¹²⁵I]Tyr⁴-Angiotensin II (2200 Ci/mmol, 81.4 TBq/mmol) was from PerkinElmer (Cambridge, UK). Primary antibodies were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany; AT₁R: sc1173, rabbit; AT₂R: sc9040, rabbit; ACE: C-20 sc-12187, goat; grp75: sc1058, goat) and New England Biolabs (Hitchin, UK; CoxI: 4850; mouse). All HSP conjugated secondary antibodies were from Thermo Scientific (Northumberland, UK). All other chemicals if not otherwise specified were from Sigma-Aldrich (UK and Switzerland).

Subcellular fractionation. Mitochondrial isolation for respirometry. Isolation of liver mitochondria was performed immediately after tissue harvesting at 4 °C using differential centrifugation⁴⁶. The excised livers were immersed in ice-cold liver isolation buffer (mannitol 220 mM, sucrose 70 mM, morpholinopropane sulfonic acid 5 mM, pH 7.4) and minced with scissors, and homogenized in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 10 minutes at 700 g. The supernatant was collected and centrifuged 3 times for 10 minutes at 7,000 g. The pellets were then used at a final concentration of 50–100 mg protein/ml. Protein concentration was determined using the Quant-iT™ protein assay kit (Qubit fluorometer, Invitrogen).

Isolation of other subcellular fractions for Western blot analysis. All isolation steps, including centrifugation were carried out at 4 °C. Livers from 12 week old male Sprague-Dawley rats were excised and placed in ice cold isolation medium (mannitol 250 mM, HEPES pH 7.4 5 mM, EGTA 0.5 mM) before mincing and washing in isolation solution supplemented with a protease inhibitor cocktail, followed by homogenization in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 g for 10 minutes resulting in the nuclear pellet and post nuclear supernatant. The supernatant was centrifuged at 10,300 g for 10 minutes resulting in the crude mitochondrial pellet and post-mitochondrial supernatant. This supernatant was then centrifuged at 25,000 g for 30 minutes giving a lysosomal pellet. All these steps were performed using a Beckman J2-MC Centrifuge with a JA-20 rotor. Finally, the supernatant was centrifuged at 100,000 g for 1 hour (Beckman 70 ultracentrifuge with a 70.1 Ti rotor) to produce the microsomal pellet and a cytosolic supernatant. All pellets were resuspended in 500 µl of isolation medium before snap freezing in liquid nitrogen and storing at –80 °C.

Isolation of pure mitochondria (PM) and mitochondrial associated membranes (MAM). A Percoll density gradient separation protocol was used to separate PM from MAM as previously described²⁴. In short, crude mitochondria were prepared as described above and then layered on a column of 30% Percoll before centrifuging at 95,000 g for 40 minutes (Beckman L-70 Ultra-Centrifuge with an SW41 rotor). The two bands (upper band MAM, lower band PM) were then further purified at 6300 g for 10 min (Beckman J2-MC Centrifuge; JA20.1 rotor) and the pellet (PM) and supernatant collected (MAM). The purified MAM were then pelleted by centrifugation at 100,000 g for 1 hr (Beckman L-70 Ultra-Centrifuge with 70.1 Ti rotor) and the pellets were resuspended in 500 µl of buffer.

Immunoprecipitation and western blotting. For immunoprecipitation of ACE, samples and antibodies were processed using Pierce Crosslink IP kit (Cat. No.26147; Thermo Scientific, Northumberland, UK), according to the recommended protocol, with all steps carried out at 4 °C and all centrifugations at low speed (3000 g) for 30–60 seconds. 10 µg ACE C-20 antibody was crosslinked to the protein A/G agarose resin and 1 mg protein was used as input. The immunoprecipitation was performed either in Tris-Buffered Saline, (TBS, 0.025 M Tris, 0.15 M NaCl; pH 7.2) or TBS supplemented with 0.5 mM EDTA, 0.5% NP-40, 2.5% glycerol to promote ACE binding.

For immunoprecipitation of AT₁R and AT₂R, samples were solubilized using ice-cold buffer (50 mM HEPES, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 30 mM PyroPO₄, 10 mM NaF, and 1 mg/ml bacitracin) and lysates were centrifuged at 14,000 rpm for 30 min. The

supernatants (400 µg of total protein) were first pre-cleared with equilibrated protein G sepharose beads (GE Healthcare, Chalfont St Giles, UK) and then incubated with 2 µg antibody overnight at 4 °C. Immune complexes were precipitated with protein G sepharose beads, washed three times with ice-cold buffer and heated at 95 °C for 5 min in loading sample buffer.

Proteins were resolved using 10% or 4–12% NuPAGE bis-tris gels (Invitrogen). Gels were transferred to nitrocellulose (Hybon-C-Super, Amersham) or PVDF membranes (Millipore, Billerica, MA, USA) using a Transblot electro-transfer apparatus (Bio-Rad). To maintain integrity of IgG at 130 kDa (Fig. 3), we used a non-denaturing 4× loading buffer without dithiothreitol and β-mercaptoethanol (200 mM Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.1% bromophenol blue). For immunoblotting, membranes were blocked with 5% BSA diluted in PBS containing 0.1% tween (PBST) and probed with specified primary antibodies in 2.5% BSA diluted in PBST. Alternatively membranes were blocked in PBS + 0.05% Tween (PBST) + 5% fat-free milk and incubated overnight in primary antibody at 4 °C. Blots were then washed and incubated with HRP-conjugated secondary antibodies (Amersham). Immunoreactive bands were detected an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

Mass spectrometry. For protein identification, samples from the immunoprecipitations, as well as purified CM, MAM and PM fractions were separated on NuPAGE 10% or 4–12% gels (Invitrogen), respectively, and stained with Coomassie blue according to standard protocols. Proteins were identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS) and MS/MS spectra search at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA, USA) as previously described⁴⁷. Proteins with three or more unique peptide matches were considered for further bioinformatics analysis.

Bioinformatic analysis. GO terms related to angiotensin were found using amiGO (<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>), all the gene names in these GO terms were also collected. The biomaRt R package (<http://www.bioconductor.org/packages/2.11/bioc/html/biomaRt.html>) was then used to find the UniProt gene names from both the GO terms and the gene names for each species. Additionally the GO terms were also used in Uniprot directly (<http://www.uniprot.org/>) to find the UniProt gene names for each species. These 3 lists were then combined to give a complete list for angiotensin genes as possible. This list was then compared to known mitochondria genes from the MitoCarta and MitoMiner databases as well as the groups identified by mass spectrometry in MAM, CM and PM fractions.

Measurement of mitochondrial respiration. Isolated mitochondria were resuspended in respiration buffer (110 mM sucrose, 0.5 mM EGTA, 3.0 mM MgCl₂, 80 mM KCl, 60 mM K-lactobionate, 10 mM KH₂PO₄, 20 mM taurine, 20 mM HEPES, 1.0 g/l BSA, pH 7.1) at a concentration of 0.8 mg/ml and incubated with the AngII (1 µM) for 20 min on ice, and respiration rates were measured at 37 °C with the High Resolution Oxygraph (OROBOROS; Oxygraph-2k, Graz, Austria). Maximal oxidative capacities were determined in the presence of saturating concentrations of oxygen, ADP (0.25 mM) and specific mitochondrial substrates. For complex I-dependent respiration, substrates were glutamate (10 mM) plus malate (5 mM). For measurement of complex II-dependent respiration, rotenone (0.5 µM) and succinate (10 mM) was used. Complex IV-dependent respiration was measured with ascorbate (4 mM) and N,N,N',N'-tetramethyl-p-phenyldiamine (TMPD, 0.5 mM). Sodium azide (5 mM) was used to measure autooxidation. Oxygen consumption is expressed as pmol × s⁻¹ × mg mitochondrial protein⁻¹.

Radioligand binding assays. Saturation binding. For saturation binding, mitochondrial homogenate preparations from n = 3 rat livers were assayed in separate experiments. Homogenates (100 µL) were incubated in assay buffer (NaH₂PO₄, 50 mM; NaCl, 100 mM; EGTA, 1 mM; MgCl₂, 10 mM; BSA, 0.2%; pH 7.4) with increasing concentrations (15 pM–2 nM) of [¹²⁵I]-AngII (in a final volume of 200 µL) for 1 h at room temperature (22 °C). Unlabelled AngII (10 µM) was used to determine non-specific binding at each concentration in duplicate tubes. Homogenates were centrifuged (20,000 g, 10 min, 4 °C) to break equilibrium, pellets re-suspended in 500 µL ice-cold Tris-HCl (50 mM, pH 7.4) buffer to remove unbound ligand and centrifuged as previously. Resulting pellets were counted in a gamma counter. Data from these experiments were analysed using the iterative non-linear curve fitting programs Kinetic and Ligand (KELL package, Elsevier Biosoft, Cambridge, U.K.) to determine the equilibrium dissociation constant (K_D) and maximal receptor density (B_{max}) and Hill slope (nH).

Fixed concentration competition assays. For fixed concentration competition assays, pooled (n = 5 rat livers) MAM homogenate preparations were individually assayed (n = 5 assays) each with 3 to 6 replicate assay points. Homogenates (100 µL) were incubated in assay buffer as previously with 0.5 nM [¹²⁵I]-AngII (in a final volume of 200 µL) for 1 h at room temperature (22 °C), with or without the addition of Angiotensin-II (10 µM) to determine non-specific binding. Additionally, either Losartan (1 µM) or PD123319 (1 µM) were included to determine presence of AT₁ vs. AT₂ receptors. Graphs were prepared to show reduction of specific binding achieved by each competing compound.



Statistical Analysis. Mitochondrial respiration rates were assessed by analysis of variance (ANOVA). The Statistical Package for Social Sciences version 18.0 (SPSS, Chicago, IL) was used for statistical analysis.

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R.A., R.B., S.D., J.A.H., R.E.K., P.S.L., J.R.A.S. and J.M.V. are co-first authors on this manuscript. A.P.D., A.J.M., J.T., S.M.J., H.M., G.S. are senior authors on this manuscript. R.A., J.R.A.S., A.P.D., A.J.M., J.T., S.M.J., H.M. and G.S. conceived and designed experiments. R.A., R.B., S.D., J.A.H., R.E.K., P.S.L., J.R.A.S., G.S. and J.M.V. performed the experiments and analyzed the data; R.A., J.R.A.S., J.T., S.D., A.P.D., H.M. and G.S. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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