Insights into the role of renin-angiotensin systems in regulating mitochondrial function

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PhD

I, Rónan Astin, confirm that the work presented in this thesis is my own. Where data has been gathered in collaboration with others, this is clearly indicated in the thesis. Where information has been derived from other sources I confirm that this has also been clearly indicated here within.

Signed:

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Abstract

The renin-angiotensin system (RAS) is an evolutionarily conserved enzyme system which plays a major role in circulatory homeostasis. This traditional concept of the RAS as a circulating enzyme system has, over the last two decades, been challenged. It is now well established that RAS exist within most tissues and operate independently of the circulating system, with the complexity of the RAS increasing as novel effector proteins, enzymes and receptors have been identified. It is now accepted that RAS can act intracellularly, and in some organs entire RAS exist within single cells. The functions of these RAS are still being elucidated, but data suggest a role in regulating metabolic efficiency.

Mitochondria are intracellular organelles responsible for producing the main energy currency of the cell – adenosine triphosphate (ATP). Understanding of their functions has expanded to include cell signalling, regulating cell cycle, and modulating metabolic function. Over the last few years, the idea that RAS may influence mitochondrial function has gained credence. Mitochondrial number, replication and function all appear to be affected by RAS activity, whilst components of the RAS have been identified in association with mitochondria raising the possibility of a mitochondrial RAS. Thus, I examined the hypothesis that a RAS exists within mitochondria.

This hypothesis was tested using four complimentary techniques. Existence of RAS proteins within the mitochondrial proteome was sought by interrogation of established databases and mass spectrometric analyses of mitochondria isolated from rat liver; no major RAS proteins were found. Attempts to identify a RAS receptor by immunochemistry failed, whilst radioligand binding studies revealed a binding site in membranes associated with mitochondria (MAM) but not mitochondria themselves.

Finally, no effect of AngII on mitochondrial respiration could be found at physiological or supra-physiological concentrations.

In conclusion, I could find no evidence of a mitochondrial RAS in rat liver suggesting the effects of the RAS on cellular metabolism are not mediated by direct interaction with mitochondria.

List of Abbreviations

ACE	Angiotensin-I converting enzyme
ACE2	Angiotensin-I converting enzyme 2
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
^{125}I-AngII	Radioiodinated AngII
AngI	Angiotensin I
AngII	Angiotensin II
AngIII	Angiotensin III
AngIV	Angiotensin IV
Ang(1-7)	Angiotensin 1-7
Ang(1-9)	Angiotensin 1-9
ANOVA	Analysis of variance
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
AT_3R	Angiotensin II type 3 receptor
AT_4R	Angiotensin II type 4 receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CoxIV	Cytochrome C oxidase subunit IV
CuZn-SOD	Copper-Zinc superoxide dismutase
DNA	Deoxyribonucleic acid
ETF:QO	Electron-transferring flavoprotein:ubiquinone oxoreductase

ETS	Electron transport system
ESI	Electrospray ionisation
FAD^+	Flavin adenine dinucleotide
$FADH_2$	Reduced form of flavin adenine dinucleotide
GCPD	Glycerophosphatase dehydrogenase
H^+	Hydrogen ion (proton)
H_2O	Water
H_2O_2	Hydrogen peroxidase
HepG2	Human hepatocellular liver carcinoma cell line
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
kDa	Kilo Daltons
Mab	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MAM	Mitochondrial associated membrane
MAPK	Mitogen activated protein kinase
Mn-SOD	Manganese-dependent isoform of superoxide dismutase
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
Na^+	Sodium ion
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate

Nox	NADPH oxidase
O_2	Oxygen
O_2•	Superoxide
$ONOO^-$	Peroxynitrite
OXPHOS	Oxidative phosphorylation
Pab	Polyclonal antibody
PKC	Protein Kinase C
PLA_2	Phospholipase A 2
PLC	Phospholipase C
PLD	Phospholipase D
RAS	Renin angiotensin system
RCR	Respiratory control ratio
ROS	Reactive oxygen species
SD	Standard deviation of the mean
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TCA	Tricarboxylic acid
UCP	Uncoupling protein
VDAC	Voltage dependent anion channel

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1. INTRODUCTION

1.1. The endocrine RAS

The circulating (endocrine) renin-angiotensin system (RAS) is an evolutionarily conserved enzyme system that plays a key role in circulatory homeostasis. In phylogenetic analyses, components of this RAS are found in primitive chordates (emerging approximately 550 million years ago) with a complete system evident by the time of the bony-fish/tetrapod divergence (approximately 400 million years ago) and evidence of horizontal transfer of RAS components to bacteria from ancestral non-vertebrate species (Fournier et al. 2012).

In the endocrine RAS, hepatically derived angiotensinogen is cleaved by the aspartyl protease renin of renal juxtaglomerular origin to yield the inactive decapeptide angiotensin I (AngI). AngI is then, in turn, cleaved by angiotensin I converting enzyme (ACE) to produce the octapeptide angiotensin II (AngII). ACE is a zinc metallopeptidase present both in circulating and endothelially bound forms, with particularly high expression on pulmonary endothelial cells. The AngII it produces is regarded as the most important effector protein of the RAS. AngII binding at the Angiotensin II Type 1 receptor (AT_1R) on vascular endothelium causes vasoconstriction whilst at the adrenal glomerulosa it stimulates the secretion of aldosterone and subsequent reabsorption of sodium and water in the renal tubules. AngII also has a direct sodium-reabsorptive effect in the kidney, and acts in the brain to stimulate thirst (Fitzsimons 1998). Finally, ACE degrades the vasodilator bradykinin thus amplifying the vasoconstrictor effects of AngII. The cumulative effect of these actions is to increase blood pressure, and the RAS is viewed as one of the most important modulators of the systemic circulatory system.

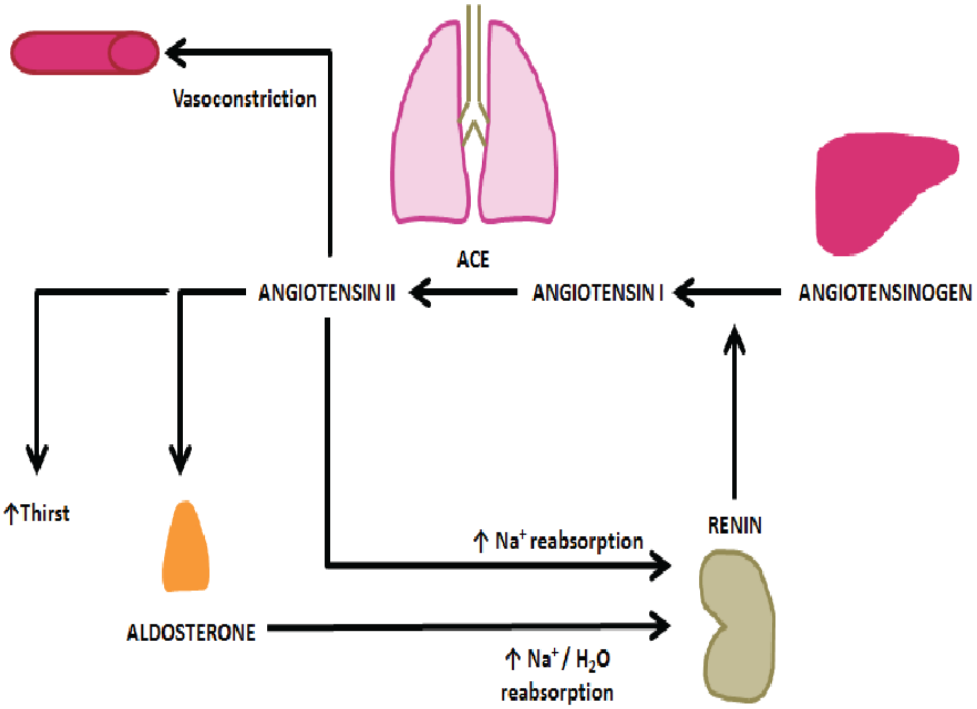

Figure 1. The endocrine RAS. Angiotensinogen released from the liver is converted to AngI by renin (of renal origin). Angiotensin I converting enzyme (ACE) converts AngI to AngII in the pulmonary bed which then acts at the vascular endothelium to cause vasoconstriction, the brain to cause thirst and the kidney to increase Na^+ reabsorption. AngII also acts at the adrenal to cause aldosterone release which then acts at the kidney to increase Na^+ and H_2O reabsorption. The combined effect of these actions is to increase circulatory volume and blood pressure.

1.2. The extended RAS

Over the last two decades, our understanding of the classical RAS has been extended by the discovery of additional components and their actions. The metabolic derivatives of AngII have been identified as important effector peptides in their own right. AngII is degraded to the heptapeptide des-Asp^1–AngII, also known as Angiotensin III (AngIII) by vascular and erythrocyte glutamyl aminopeptidase A (Ward et al. 1990; Chauvel et al. 1994) and subsequently to the hexapeptide des-Asp^1, des-Arg^2-AngII, also known as angiotensin IV (AngIV) by alanyl aminopeptidase N. Both AngIII and AngIV are active peptides with similar (but less potent) circulatory effects to those of AngII. Angiotensin (1-12) (Ang(1-12)) is a recently discovered peptide with a two amino acid extension at the C-terminus of AngI (Nagata et al. 2006) which is formed independently of renin action but is thought to be a peptide precursor of AngII.

Further, there is redundancy in the conversion of AngI to AngII, with numerous other enzymes carrying out the same cleavage. Chymase catalyses the same reaction in many tissues (Singh, Le, et al. 2008; Cristovam et al. 2008; Tom et al. 2003) whilst members of the caspase family of proteins and cathepsin G also perform this function (Urata et al. 1990; Naruse & Inagami 1982; Lorenz 2010).

Alongside the AngI-ACE-AngII-AT_1R pathway, a second seemingly counter-regulatory arm of the endocrine RAS has been identified. ACE2 is a homologue of ACE and yields the heptapeptide angiotensin (1-7) (Ang(1-7)) from AngII (Vickers et al. 2002) whilst also degrading AngI to the inactive nonapeptide angiotensin (1-9) (Ang(1-9)). Ang(1-9) is then converted to Ang(1-7) by ACE (Tipnis et al. 2000). Ang(1-7) acts at the Mas receptor to produce effects that are largely opposite to those mediated by the AT_1R (Santos et al. 2013a) thus acting as a counterbalance to the classical system. A further counter-regulatory pathway is effected by an alternative AngII receptor, the Angiotensin

II Type 2 receptor (AT_2R) whose role, while less well understood than that of the AT_1R, seems widely to be in opposition (Carey & Siragy 2003).

Along with the discovery of several novel components of the RAS, additional actions of existing components are also being elucidated. Most notable of these was the discovery that both prorenin and renin can act as ligands at the recently identified (Pro)renin receptor, implying that prorenin and renin can exert effects independent of AngII (Nguyen et al. 2002; Nguyen et al. 1996). The complexity of the RAS continues to increase with the identification of further components, such as Angiotensin A and Alamandine (Carey 2013; Carey & Siragy 2003).

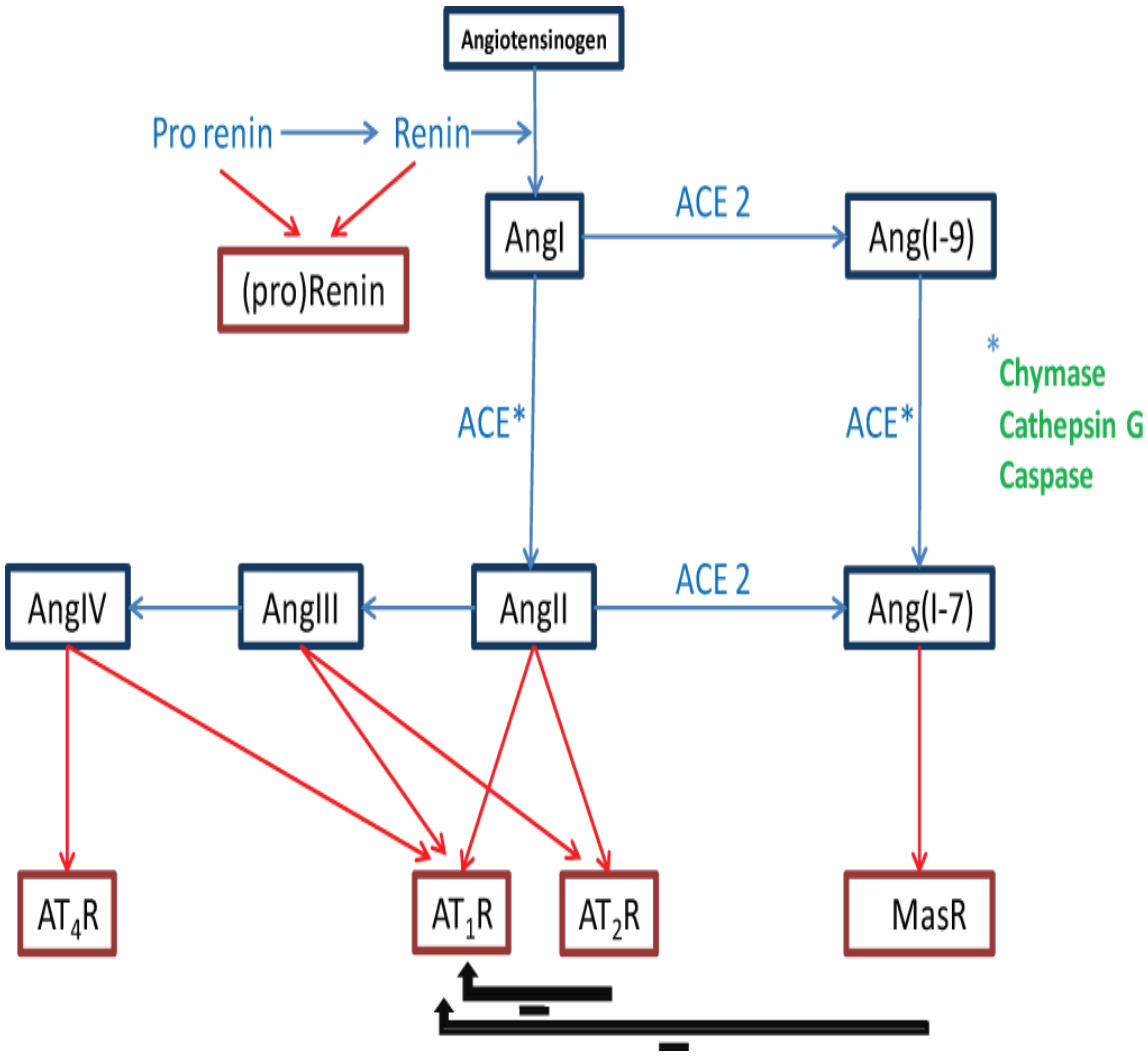

Figure 2. The extended RAS. As well as its action at AT_1R, AngII acts at AT_2R to have largely counter active effects. AngII is metabolised to AngIII then AngIV, both metabolically active peptides. AngIV acts at both AT4R and AT1R whilst AngIII acts predominantly at AT_1R and AT_2R. Further counter-regulation is achieved through the action of Ang(1-7), formed by ACE2 metabolism of AngII, at the Mas receptor. There is redundancy in the action of ACE; chymase, cathepsin G and caspase can all catalyse the same reactions. The recent discovery of renin and pro renin action at the (pro)renin receptor provides an action of the RAS independent of Angiotensin peptide action.

1.3. RAS receptors

AngII exerts its effect through action at a set of AngII receptors. There are three main receptors in humans; AT_1R, AT_2R and AT_4R. The latter binds AngIV with high affinity but AngII and AngIII with low affinity and is also capable of binding multiple other peptides. The receptor has been identified as an insulin-regulated membrane aminopeptidase (IRAP) (Chai et al. 2004; Demaegdt et al. 2009) and its expression confirmed in various mammalian tissues including adrenal gland, bladder, colon, kidney, heart, prostate, brain and spinal cord (Zhuo et al. 2013; Wright et al. 1995). However, the physiological roles of AT_4R are not well understood. Further, its ligand (AngIV) mediates many of its known effects through AT_1R (Yang et al. 2010; Yang et al. 2008) or AT_2R (Vinh, Widdop, Drummond, et al. 2008; Vinh, Widdop, Chai, et al. 2008).

By far the more abundant receptors are the AT_1R and AT_2R. Both are G protein coupled seven hydrophobic transmembrane glycoprotein receptors which share 34% sequence homology. The AT_1R is ubiquitous, whilst the AT_2R is being identified in an increasing number of tissue types, although its mechanisms of action are less well understood.

1.3.1. AT_1R

Although the AT_1R has been sequenced, its crystal structure has not been solved (Matsoukas et al. 2013). That said, a combination of computer modelling and AngII binding studies have elucidated the relative binding affinity of ligands at AT_1R as AngII > AngIII > AngIV > Ang(1-7) (Bosnyak et al. 2011). Ligand binding at AT_1R leads to activation of several signalling cascades. These can roughly be divided into G protein coupled and non-G protein coupled pathways. On AngII binding, AT_1R interacts with G protein complexes which activate downstream effector peptides including protein kinase C (PKC), phospholipase C (PLC), phospholipase A_2 (PLA_2) and phospholipase

D (PLD) (Ushio-Fukai et al. 1999). Activation of PLC seems of particular importance, since it leads to inositol-1,4,5-triphosphate (IP_3) generation within seconds (along with diacyl glycerol, DAG) which acts on its receptor (IP_3R) on endoplasmic reticulum to cause Ca^{2+} release (Marks 1997). Other G protein linked signalling pathways enhance growth, migration and inflammation related pathways including PKC mediated activation of NAD(P)H oxidase (Nox)- a major source of cellular reactive oxygen species (ROS) (Montezano et al. 2014; Dikalov & Nazarewicz 2013; Bataller et al. 2003).

Along with G proteins, AT_1Rs also activate the mitogen-activated protein kinases (MAPKs) which play essential roles in gene expression, protein synthesis, metabolism and growth (Palomeque et al. 2009; Smith et al. 1999). This involves the ERK 1/2, JNK and p38 MAPK pathways, all of which can be activated downstream of AT_1R (Mehta & Griendling 2007). Add to these pathways the receptor and non-receptor tyrosine kinase pathways, and the complexity of AT_1R signalling becomes apparent (Yaghini et al. 2007; Gasparo et al. 2000; Hunyady & Catt 2006; Wang et al. 2004).

Downstream of these signalling cascades, AT_1R activation leads to cellular changes stimulating growth, inflammation, oxidative stress and vasoconstriction (Cassis et al. 2010).

1.3.2. AT_2R

As well as binding AT_1R, AngII binds AT_2R with high affinity and in fact displays slight preferential selectivity for AT_2R (Bosnyak et al. 2011). The affinity binding hierarchy of AT_2R follows AngII > AngIII > AngIV > Ang-(1-7), with all showing selectivity for AT_2R over AT_1R (Bosnyak et al. 2011). The AT_2R is ubiquitously expressed in foetal tissue but is down-regulated after birth (Nahmias & Strosberg 1995) with evidence suggesting

it may be up-regulated in disease states; in cadaveric studies of human heart failure the relative abundance of AT_2R is increased (Haywood et al. 1997). Signalling pathways of the AT_2R are poorly described but there is evidence that stimulation of AT_2R triggers an autocrine cascade including tyrosine phosphatase, serine/threonine phosphatase and bradykinin/nitric oxide/cyclic GMP axis activation (Carey et al. 2000; Siragy 2000). Interestingly, evidence suggests that AT_2R can directly bind to (and inhibit) the AT_1R, an action which does not necessarily depend on AT_2R activation (AbdAlla et al. 2001).

1.3.3. Mas receptor

Ang(1-7) displays the lowest binding affinity at AT_1R and AT_2R of all the established RAS ligands (Bosnyak et al. 2011), instead exerting its biological influence through action at the Mas receptor. Like the other AngII receptors, Mas is a G protein coupled receptor. It is encoded by the MAS oncogene, and expression has been proven in most tissues (Xu et al. 2011; Metzger et al. 1995; Alenina et al. 2008). A growing evidence base suggests that the ACE2/Ang(1-7)/Mas axis counteracts most of the effects of the ACE/AngII/AT_1R axis (Ferreira & Santos 2005). In cardiac and endothelial tissue, Mas activation causes vasodilatation possibly through PI3-AKT signaling to cause release of NO and prostaglandins (Walkyria Sampaio et al. 2007; Santos et al. 2006), whilst in the kidney, expression is increased in renal ischaemia (Zimmerman & Burns 2012) and counteracts MAPK singalling at AT_1R (W Sampaio et al. 2007). With evidence for antifibrotic and antiproliferative effects of Mas activation accumulating (Wu et al. 2014; Santos et al. 2013b) the position of the ACE2/Ang(1-7)/Mas axis as a counter regulatory mechanism to AT_1R activation strengthens.

1.4. Tissue RAS

As well as the circulating RAS, independently regulated RAS exist and operate at the tissue level (Bader & Ganten 2008; Velez 2009). Such RAS appear ubiquitous, having been identified in (amongst many other organs and tissues) kidneys, liver, spleen, pancreas, heart, lung, adrenal glands, brain, immune system and skeletal muscle (Montgomery et al. 2003; Paul et al. 2006; Jones & Woods 2003; Sakai & Sigmund 2005; Bader & Ganten 2008; Campbell & Habener 1987; Campbell & Habener 1986). The concept of a tissue RAS gained credence with the identification of RAS component expression in tissues distant to their classical site of production or action, such as the 'renal' enzyme renin in brain tissues (Ganten et al. 1971). The mapping of AngII receptors at sites in the brain that cannot be reached by circulating AngII due to the blood-brain barrier (Phillips et al. 1977; Ganten et al. 1971) further supported the idea of a sequestered tissue RAS. Similarly, in the kidney the localisation of renin messenger RNA (mRNA) and protein outwith juxtaglomerular cells and within the proximal tubules and the collecting duct system (Moe et al. 1993), together with confirmation of angiotensinogen and ACE expression within the kidney (Schulz et al. 1988; Gomez et al. 1988), pointed to a renal RAS that could function independently of the circulating system.

The completeness of tissue RAS differs between organs. Some tissues have the full enzymatic cascade, including tissue specific enzyme isoforms (Sinn & Sigmund 2000) producing biologically active AngII, AngIII, Ang(1-7) and AngIV, whilst others depend on the uptake of key components from the circulation (van Kesteren et al. 1997; Krop & Danser 2008; Danser 2003). For example, in the heart, AT_1R and AT_2R are expressed but local AngII production is dependent on the uptake of renin from the circulation (Peters et al. 2002) or renin released from cardiac mast cells (Mackins et al. 2006).

Within the tissue on a cellular level, AngII produced intracellularly can be secreted to act via plasma membrane AngII receptors of neighbouring cells (paracrine actions) or on the synthesising cell itself (autocrine action) (Inagami et al. 1990; Inagami et al. 1986; Sadoshima et al. 1993; Vila-Porcile & Corvol 1998). In this way, the RAS can influence single cell activity or local tissue activity, leading to whole organ physiological changes.

The roles of tissue RAS are still being elucidated but are extensive. AngII acts as a cellular growth factor in vascular and cardiac tissues (Imanishi et al. 2004; Vukelic & Griendling 2014) whilst causing hypertrophy in cardiac and skeletal muscle (AngII being essential for transforming load into growth) (Gordon et al. 2001; Lijnen & Petrov 1999; Sadoshima et al. 1993). It regulates fibroblast function, with a pro-fibrotic role proven in kidney, liver, lung and cardiac tissue amongst others (Granzow et al. 2014; Mezzano et al. 2001; Wynn 2008; Sadoshima & Izumo 1993; Königshoff et al. 2007) and influences cell differentiation such as that of preadipocyte to adipocyte (Darimont et al. 1994; Janke et al. 2002) as well as vascular and bone progenitor cells (Roks et al. 2011; Durik et al. 2012). AngII also regulates cell cycle, being an important pro-apoptotic factor in cardiac and alveolar cells (Wang et al. 1999; Papp et al. 2002; Schröder et al. 2006). These actions are predominantly mediated via the AT_1R. Action of AngII at the AT_2R seem broadly antagonistic to these effects; cardiac hypertrophy is inhibited (Jones et al. 2004), it mediates antifibrotic signalling (Schulman & Raij 2008) and, in the kidney, vasodilatation and natiuresis is stimulated (Carey et al. 2000; Nouet & Nahmias 2000).

These multiple cellular actions of tissue RAS explain how the accepted benefits of ACE inhibitor and AngII receptor blocker medication in hypertension, cardiovascular disease and diabetes appear to be independent of their blood pressure lowering actions (Pfeffer

et al. 1992; Dahlöf et al. 1998; Park et al. 2000; Opie 2001). Modulating tissue RAS function therefore offers a potentially powerful way of counteracting organ dysfunction.

1.5. Intracrine RAS

The paracrine and autocrine actions of AngII are mediated by action at the plasma membrane receptors. However, over the last 20 years, evidence of intracellular AngII activity has accumulated. The existence of intracellular AngII receptors emerged with nuclear AT_1Rs demonstrated in the renal cortex and medulla, vascular smooth muscle, cardiomyocytes and hepatocytes amongst others (Julia L Cook et al. 2006; Eggena et al. 1993; Pendergrass et al. 2006; Tadevosyan et al. 2010). Intracellular microinjection of AngII in proximal tubule cells caused an increase in intracellular Ca^{2+} (iCa^{2+}) peaking at 30 seconds proving these receptors to be functional (Zhuo et al. 2006). This effect was attributed to intracellular AngII action since blockade of plasma membrane receptors with losartan (a specific AT_1R antagonist) had no impact whilst microinjection of AngII and losartan abolished the iCa^{2+} response (Zhuo, Li, et al. 2006). Since then, AngII binding at the nucleus has also been shown to induce ROS production within the nucleus (Gwathmey et al. 2010; Pendergrass et al. 2009) and directly induce transcription of factors including transforming growth factor beta (TGF-β), macrophage chemoattractant protein-1 (MCP-1) (X. C. Li and Zhuo 2008b), cyclic AMP response element binding protein (CREB) (Julia L Cook et al. 2006) and platelet derived growth factor (PDGF) (J. L. Cook, Zhang, and Re 2001; Julia L Cook et al. 2002), NFκB (Tadevosyan et al. 2010), as well as RAS components such as renin and angiotensinogen (Eggena et al. 1993).

How AngII, its receptors and other components of the RAS come to reside intracellularly is a matter of debate. There is abundant evidence for AngII-AT_1R complex internalisation following binding on the plasma membrane (Zhuo & Li 2007; Li et al. 2009; Li et al. 2007; Li & Zhuo 2008) with the suggestion that it is these internalised components that form the ‘intracellular’ RAS. Certainly, there is evidence of

ongoing receptor activity in endocytosed vesicles (Murphy et al. 2009; Cook & Re 2012), and also of AngII 'spill' into the cytoplasm (Cho et al. 2003; Brown et al. 1982).

Alternatively, AngII may be produced entirely within the cell and act independently of plasma membrane receptors. In support of this, in neonatal rat ventricular myocytes (NRVMs) exposed to high glucose serum, AngII accumulates intracellularly despite blockade of plasma membrane AT_1R with candesartan (Singh, Baker, et al. 2008; Singh et al. 2007). Further, the components of the RAS required to produce AngII have all been localised within single cells (Naruse et al. 1982; Inagami et al. 1986; Inagami et al. 1990). A truncated renin transcript lacking the coding sequence to target the protein to the secretory pathway (exon(1-9)) has been identified and shown to produce a renin isoform that remains in the cytoplasm (Peters 2002; Lavoie et al. 2004; Lee-Kirsch et al. 1999; Sinn & Sigmund 2000) whilst angiotensinogen mRNA expression has been found in multiple tissues (Campbell & Habener 1987; Campbell & Habener 1989; Campbell & Habener 1986; Tadevosyan et al. 2010; Dzau et al. 1987; Lee et al. 1987) including an isoform that can be retained within the cell depending on glycosylation state (Sherrod et al. 2005). Finally, transfecting cells with a non-secreted form of angiotensinogen causes an increase in intracellular AngII, proving potential for intracrine AngII generation (Cook & Re 2009).

It appears that the control of intracellular RAS is independent of circulating systems. Adrenal intracellular renin is increased in response to elevated serum potassium whereas circulating renin is decreased (Nakamaru et al. 1985), whilst high glucose causes a 10-fold increase in intracellular ACE but has no effect on extracellular ACE (Cristovam et al. 2008). Such an enclosed, internally regulated system would offer an elegant way of modulating cell activity.

1.6. RAS and physical performance

Tissue RAS appear to influence aspects of physical performance. Whilst polymorphic variations exist in the genes of many of the RAS components, the most studied is that found in the human ACE gene. The absence (deletion [D]) rather than the presence (insertion [I]) of a 287-base pair *Alu* repeat sequence within intron 16 of the ACE gene is associated with higher circulating plasma (Rigat et al. 1990) and tissue (Costerousse et al. 1993; Danser et al. 1995) ACE activity. Numerous studies have shown an association of the I allele with endurance exercise. An excess of the I allele has been found in distance runners (Myerson et al. 1999), elite ultra-long distance swimmers (Tsianos et al. 2004) and Olympic rowers (Gayagay et al. 1998). This relationship seems to relate to metabolic efficiency; training-related improvement in efficiency of muscle contraction in Caucasian military recruits measured by delta efficiency (DE; percentage ratio of change in work performed per minute to the change in energy expended per minute) was strongly genotype dependent, with only II individuals (and not DD) showing a significant increase in DE (Williams et al. 2000). Furthermore, amongst Caucasian military recruits undergoing training, the I allele was associated with relative conservation of fat stores (Montgomery et al. 1999). As one would expect, this greater metabolic efficiency seems to translate to better performance in hypoxic conditions (Tsianos et al. 2006; Montgomery et al. 1998; Kalson et al. 2009; Tsianos et al. 2005). British elite mountaineers demonstrate an allele skew towards the I allele (Woods & Montgomery 2001), whilst in 139 mountaineers attempting to ascend to 8000m, maximum altitude achieved was significantly associated with the same allele (Thompson et al. 2007).

These data seem to hint at a role for tissue and intracellular RAS in modulating cellular energetics.

1.7. RAS and cellular energetics

Some of the first evidence of a role for RAS in cellular energetics came from studies of tissue oxygen uptake in organs perfused with AngII. AngII caused a dose dependent increase in oxygen uptake in the perfused rat hindlimb (Colquhoun et al. 1988) and liver lobules (Matsumura et al. 1992) whilst in the kidney AngII perfusion was associated with increased oxygen consumption (QO_2) and reduction in the renal efficiency for transporting sodium (QO_2/TNa^+) (Welch et al. 2005; Laycock et al. 1998; Deng et al. 2005). More recently it has been shown that inhibition of RAS (with combined ACE inhibition and AT_1R antagonism) can normalise QO_2/TNa^+ (Deng et al. 2009). In cardiac ischaemia-reperfusion injury models, higher ACE expression impairs myocardial tolerance to ischaemia (Messadi et al. 2010) whilst ACE inhibition potentiates the ischaemia-sparing effect of cardiac pre-conditioning (Miki et al. 1996; Jaberansari et al. 2001). Meanwhile inhibition of the RAS in animal models of heart failure maintains myocardial maximal oxidative capacity and adenosine triphosphate (ATP) production (Sanbe et al. 1995).

There is evidence that these effects relate to reactive oxygen species (ROS) production. In vascular smooth muscle cells (VSMC) ROS were released within minutes of exposure to AngII (Sorescu & Griendling 2002) whilst in cardiomyocytes from diabetic rats, increased AngII levels were associated with increased superoxide production (Singh, Le, et al. 2008). Causation is suggested by data showing renin inhibition to prevent the ROS accumulation. There are two major sites of ROS production in most cell types; the NAD(P)H oxidases (the Nox protein family) and mitochondria. Whilst pathways of AngII mediated signalling via cell membrane or cytosolic Nox are described (Dikalov & Nazarewicz 2013), a direct action of AngII on mitochondria has not be ruled out.

1.8. Mitochondrial physiology

Mitochondria are the primary sites of 'energy currency' production (ATP synthesis). Each mitochondrion consists of phospholipid outer and inner membranes separated by an intermembrane space. The inner membrane is folded into ridges (or cristae) and bounds the mitochondrial matrix. Within the inner mitochondrial membrane sit the protein complexes of the electron transport system (ETS) which drives ATP synthesis through oxidative phosphorylation (OXPHOS).

1.8.1. The electron transport system

The ETS is classically described as consisting of four separate complexes (complexes I-IV) and two mobile electron carriers (coenzyme Q and cytochrome C) along with ATP synthase (sometimes referred to as complex V). Each complex is a large protein aggregate composed of multiple subunits encoded by both mitochondrial and nuclear DNA (other than complex II which is encoded by nuclear DNA only). Electrons from reduced nicotinamide adenine dinucleotide (NADH) enter the ETS at complex I whilst reduced flavin adenine dinucleotide ($FADH_2$) donates electrons to complex II. As electrons pass through complexes I, III and IV, a drop in their redox potential is coupled to the extrusion of protons from the matrix across the inner mitochondrial membrane to the intermembrane space generating an electrochemical gradient. Complex IV is the terminal oxidase of the mitochondrial ETS, reducing oxygen with four electrons to produce two molecules of water. The return of the hydrogen ions across the membrane at complex V drives the synthesis of ATP.

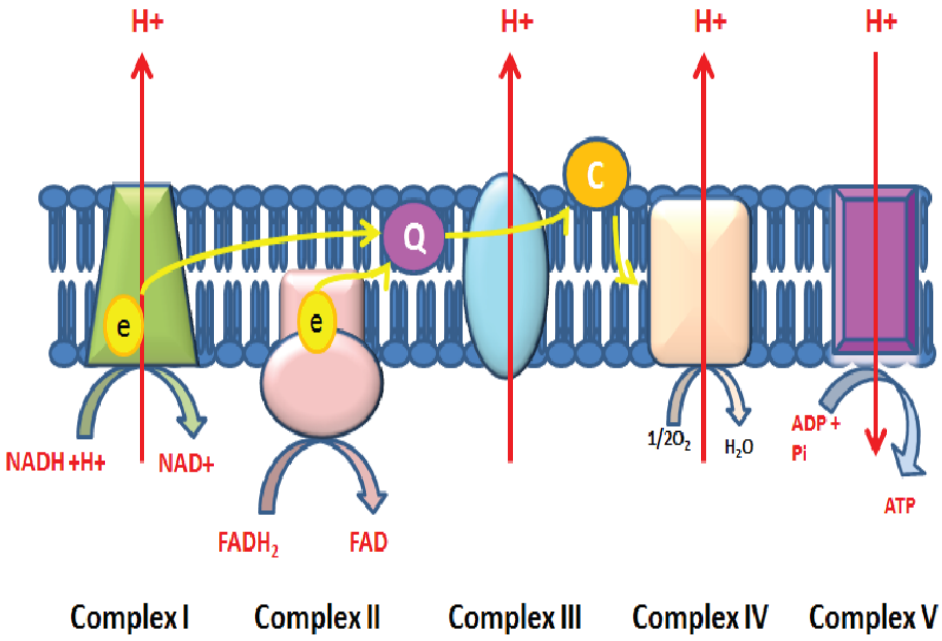

Figure 3. The electron transfer system (ETS). Electrons donated from NADH are accepted by complex I and those donated from $FADH_2$ are accepted by complex II. Electrons pass through complexes III and IV with extrusion of protons (H^+) at complexes I, III and IV. The proton motive force generated is then used by ATPase to produce ATP. Q; coenzyme Q, C; cytochrome C.

It is important to note this representation of the ETS is a simplification. More accurately it consists of six rather than four protein aggregates, with electron-transferring flavoprotein:ubiquinone oxoreductase (ETF:QO) and glycerophosphate dehydrogenase (GCPD) both residing in the inner mitochondrial membrane and contributing electrons to the ubiquinone pool and in so doing play a significant role in OXPHOS. Crucially, these complexes do not form a chain. Rather, electrons from complex I and complex II are both carried by coenzyme Q to complex III. Thus a convergent structure of electron flow to the 'Q-junction' in the ETS can be described (Gnaiger 2009) (Fig. 4). From there they pass through complex III to IV via cytochrome C. The tight coupling between electron transport and proton extrusions means that the rate of mitochondrial oxygen

consumption is an accurate measure of the total proton current (Brand & Nicholls 2011).

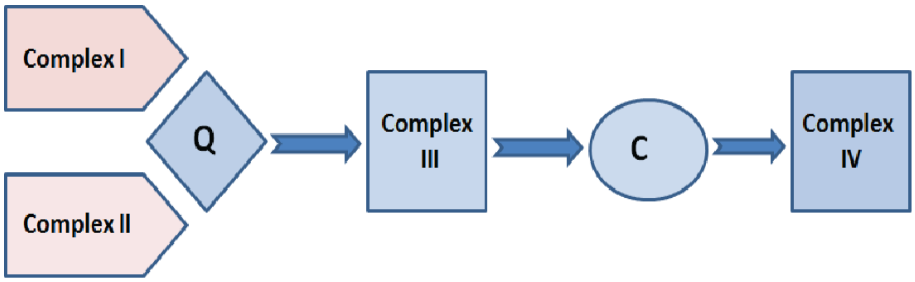

Figure 4. The convergent structure of electron flow. Electrons donated to complex I and complex II both flow to complex III via coenzyme Q (Q). From there electrons are ferried to complex IV by cytochrome C (C).

1.8.2. Metabolic substrates

The electrons required to support OXPHOS are donated by reducing equivalents produced within the cell by metabolism of energy substrates. Through glycolysis, glucose is converted to pyruvate in the cytosol which is transported to the mitochondrial matrix where it is converted to acetyl-CoA by pyruvate dehydrogenase and enters the tricarboxylic acid (TCA) cycle (also known as citric acid or Krebs cycle) producing three NADH molecules and one $FADH_2$ molecule.

Fatty acids are metabolised first in the cytosol producing acyl-CoA which is transported to the mitochondrial matrix whereupon it undergoes sequential oxidation of its carbon double bonds (β-oxidation). Each carbon bond oxidised produces one molecule each of NADH and $FADH_2$ which are fed into the ETS, along with one molecule of acetyl-CoA which enters the TCA cycle.

Amino acids can also act as substrates. Following removal of the amino group (deamination) the carbon skeletons are used to produce metabolic intermediates of the

TCA cycle. Amino acids that are degraded to acetyl-CoA or acetoacetyl-CoA are termed ketogenic because they can lead to ketone body production, whilst those that are metabolised to form pyruvate, α-ketoglutarate, succinyl-CoA, fumarate or oxaloacetate are termed glucogenic amino acids.

1.8.3. Mitochondrial respiration, coupling and efficiency

1.8.3.1. Respiratory states

The conventional description of mitochondrial bioenergetics was made by Britton Chance and Ron Williams in 1956 (Chance & Williams 1956) as a way of describing the various activity states of the ETS during experimental *in vitro* respiration. Depending on the presence of respiratory substrates and availability of ADP, four states can be described (1,2,3 and 4). State 1 refers to mitochondria respiring on their endogenous substrates in the presence of oxygen but the absence of supplemented ADP. Once these endogenous substrates are oxidised, mitochondria enter State 2, respiring at a residual or basal rate in compensation for proton leak across the inner mitochondrial membrane. State 3 is the rate of respiration achieved in the presence of saturating concentrations of substrates and ADP and is close to maximal oxidative capacity. State 4 describes the resting state of mitochondria, achieved following consumption of the exogenous ADP.

By measuring the rate of oxygen consumption during these states of activity, statements regarding mitochondrial respiratory capacity and efficiency can be made.

1.8.3.2. LEAK respiration

LEAK respiration refers to the imperfect coupling of proton movement and ATP generation. All mitochondria display endogenous proton leak across their inner membranes (Nicholls 1977). In the resting state, dissipation of the membrane potential

caused by this proton movement causes activation of the ETS in order to maintain a high membrane potential -reflected in ongoing oxygen consumption in the absence of ADP.

There are several suggested mechanisms to explain leak. Proton slip refers to a property of the proton pumps (complexes I, III and IV) whereby protons slip back to the matrix side of the membrane instead of across it during electron transfer. In addition, some protons are moved across the membrane independent of OXPHOS in the cycling of Ca^{2+} (Nicholls 2005) and the shuttling of ATP and ADP by adenine nucleotide translocase (ANT) (Brand et al. 2005).

There is also evidence that specific proteins, known as uncoupling proteins, contribute to LEAK respiration. In brown fat, uncoupling protein 1 (UCP1) functions to dissipate membrane potential with the energy that is derived from the oxidised substrates released as heat (Nedergaard & Cannon 2010). The primary role of UCP1 seems to be thermogenesis, though regulation of ROS production has also been suggested (Clarke & Porter 2013; Dlasková et al. 2010). Since UCP1 was identified, uncoupling functions of other proteins have been suggested (most notably UCP2 and UCP3). These proteins are all members of the anion carrier protein family and are located in the inner mitochondrial membrane. *In vitro* experiments have suggested that they can uncouple OXPHOS when suitably activated by superoxide and alkenals (Echtay et al. 2002; Brand et al. 2004; Brand & Esteves 2005). A role in controlling ROS production, and even ROS signalling with so called ‘mild uncoupling’ has gained credence (Echtay 2007; Divakaruni & Brand 2011), though the physiological credibility of this theory has recently been questioned (Shabalina & Nedergaard 2011).

1.8.3.3. Mitochondrial ROS

Whilst the vast majority of O_2 within in a cell is converted to H_2O at complex IV of the ETS, approximately 1-2% of cellular O_2 is consumed by the production of reactive oxygen species (ROS). ROS are produced when electrons are donated to O_2 to form superoxide (O_2^-). This can occur at various locations in the cell such as cell membrane NAD(P)H oxidase or cytosolic xanthine oxidase (Iuchi et al. 2003) but mitochondria also represent a major source of ROS (Chandel et al. 1998; Jastroch et al. 2010). Within mitochondria, ROS are predominantly produced at complex I and complex III (Turrens 2003; Li et al. 1999) though under certain pathological conditions, complex II can contribute to ROS production by directing electrons though complex I rather than complex III ('reverse electron transport') (Chouchani et al. 2014; Grivennikova & Vinogradov 2006). Other mitochondrial sources of ROS include matrix enzymes (Messner 2002; Starkov et al. 2004), outer membrane monoaminoxidases (Cadenas & Davies 2000) and mitochondrial NOS uncoupling (Brodsky et al. 2002).

Produced at ETS complexes, O_2^- can be then be released to either side of the inner mitochondrial membrane (Muller et al. 2004) where it is rapidly converted to hydrogen peroxide (H_2O_2) either by abundant superoxide dismutase (mn-SOD) in the matrix (Andreyev et al. 2005) or copper/zinc containing SOD (Cu,Zn-SOD) in the intermembrane space (Okado-Matsumoto & Fridovich 2001). H_2O_2 diffuses freely across the outer mitochondrial membrane and into cytosol.

Conditions of excess ROS production (oxidative stress) are linked to a multitude of disease processes including cardiovascular diseases, diabetes, chronic kidney disease and cancer (Rochette et al. 2014; Csányi & Miller 2014; Gwinner & Gröne 2000; Cerutti & Trump 1991). However, under non-pathological conditions ROS act as signalling molecules, oxidising redox-sensitive phosphatases/kinases and modifying

phosphorylation status of transcription factors or receptors (de Cavanagh et al. 2007; Suzuki et al. 1997). These ROS signals are suggested to play important roles in metabolism, cell differentiation, the stress response and aging (Hou et al. 2014).

1.9. The mitochondrial environment

Mitochondria exert an influence on, and are influenced by, their surrounding cellular environment (Raturi & Simmen 2013). Rather than operating as isolated units, they interact with each other and neighbouring organelles. The significance of the interaction between mitochondria and endoplasmic reticulum (ER) is of increasing interest (Hayashi et al. 2009; Raturi & Simmen 2013; Schon & Area-Gomez 2013).

Areas of the ER in close contact with the outer mitochondrial membrane (OMM) are termed the mitochondria-associated membrane (MAM). It is estimated that between 5-20% of mitochondrial membrane is in close contact with the ER at any time (Rizzuto et al. 1998). These areas appear to be regulated separately to the rest of the ER, with local enrichment in functionally diverse enzymes, involved in lipid transport, Ca^{2+} handling and other signal transductions (Hayashi et al. 2009; Bravo et al. 2011). These proteins can interact directly with the OMM since the distance between MAM and mitochondrion can be as little as 10-20 nm (Csordás et al. 2006). Communication between mitochondria and MAM has been shown to influence lipid transport (Fujimoto & Hayashi 2011), Ca^{2+} signalling and homeostasis (Szabadkai & Duchen 2008), mitochondrial fission (Marchi et al. 2014) energy metabolism (Cárdenas et al. 2010) and cellular survival (Giacomello et al. 2007).

Ca^{2+} signalling at the MAM is perhaps best described (Voelker 2005; Vance 2003; Rizzuto et al. 2004; Hajnóczky et al. 2003). In the ER, stimulation of IP_3R by IP_3 leads to a massive release of Ca^{2+} (Fieni et al. 2012; Kirichok et al. 2004; Wojtczak et al. 2010). IP_3R are highly compartmentalised in MAM (Mendes et al. 2005) leading to microdomains of high [Ca^{2+}] when stimulated. Direct interaction of IP_3R with the voltage dependent anion channel (VDAC) of the OMM leads to Ca^{2+} uptake (Szabadkai et al. 2006) whilst the high [Ca^{2+}] activates the Ca^{2+} uniporter of the IMM enabling transfer of

Ca^{2+} into the matrix (Wojtczak et al. 2010; Szabadkai & Duchen 2008; Rizzuto et al. 2009).

Increasing [Ca^{2+}] in the mitochondrial matrix can influence bioenergetics in various ways. Three TCA cycle enzymes (pyruvate dehydrogenase, α-ketoglutarate dehydrogensase and isocitrate dehydrogenase) have Ca^{2+} binding sites that regulate their activity (Rutter & Denton 1988; McCormack & Denton 1979; Denton 2009; Denton et al. 1980) whilst both glutamate and aspartate carriers are Ca^{2+} sensitive (Lasorsa et al. 2003). Ca^{2+} also modulates intramitochondrial antioxidant levels (Hopper et al. 2006). Therefore, IP_3R activation on the MAM can directly influence mitochondrial function.

The importance of MAM-mitochondrial interactions is only just being elucidated. Disruption of these interactions has been implicated in Alzheimer's and other neurodegenerative diseases (Schon & Area-Gomez 2013), insulin dependent diabetes (Tubbs et al. 2014), cardiac ischaemia-reperfusion injury (Paillard et al. 2013), myopathies (Eisner et al. 2013) and the pathogenesis of some viruses (Vance 2014).

1.10. RAS and mitochondria

Evidence that RAS influence aspects of mitochondrial function does exist, but is limited and yet to be drawn into a coherent narrative. Studies linking RAS and mitochondrial dysfunction can be broadly separated into those suggesting an effect on biogenesis and those implying an alteration in respiratory function.

When muscle is exposed to AngII in either cell culture or animal models, there is a decrease in mitochondrial number and an associated decrease in markers of mitochondrial biogenesis (Mitsuishi et al. 2009). Examination of the cardiac transcriptome of mice exposed to AngII revealed a decreased expression of important mitochondrial mRNAs including those coding for proteins integral to the ETS, beta oxidation pathway and the TCA cycle (Larkin et al. 2004). Meanwhile, chronic antagonism of RAS in mice (by ACE inhibition or AngII receptor blockade/knockout) prevents the recognised age related decrease in mitochondrial number in both kidney (Ferder et al. 2002; de Cavanagh et al. 2003) and liver (de Cavanagh, Flores, et al. 2008; Cassis et al. 2010) whilst AngII receptor blockade induces mitochondrial biogenesis in rat hearts (Mervaala et al. 2010). These data suggest RAS operates through nuclear regulatory pathways to influence mitochondrial number and ETS protein content.

There is evidence that the RAS might affect mitochondrial respiration more directly. Mitochondria isolated from bovine endothelial cells cultured in the presence of AngII display an increased State 4 respiration and decreased State 3 respiration along with a decreased resting mitochondrial membrane potential (Doughan et al. 2008). These cells also showed elevated levels of H_2O_2. Similarly, increased ROS levels were demonstrated in VSMCs exposed to AngII in association with reduction in mitochondrial membrane potential, an effect seemingly caused by interaction with mitochondrial ATP

sensitive K^+ channels (Kimura et al. 2005). In mitochondria isolated from hindlimb skeletal muscle of mice dosed with AngII for 7 days, again there was an increased State 4 respiration (Inoue et al. 2012). In these mitochondria, there was significant impairment of respiration through complex I and complex III together with an increased O_2^- production and expression of UCP3 (Inoue et al. 2012). Meanwhile, inhibition of the RAS with a non-antihypertensive dose of the ACE-inhibitor enalapril increased ETS enzyme activities and mitochondrial antioxidant levels in spontaneously hypertensive rats (Piotrkowski et al. 2009).

Many of these effects appear to be orchestrated by action of AngII at the AT_1R. In cardiac mitochondria isolated from mouse hearts subjected to acute ischaemia, prior perfusion with valsartan (AT_1R antagonist) preserved mitochondrial function (higher State 3 and State 4 respiration, preserved mitochondrial membrane potential, higher ATP/ADP ratio and shorter phosphorylation lag time) (Monteiro et al. 2005). Similarly AT_1R blockade with losartan preserves renal mitochondrial function in the spontaneously hypertensive rat (de Cavanagh et al. 2006) and streptozotocin-induced Type 1 diabetic rat – an effect associated with increased UCP2 expression and elevated cytochrome C activity (de Cavanagh, Ferder, et al. 2008).

All of these suggested effects of AngII could be mediated by activation of AngII receptors on the cell surface or nuclear membrane, with downstream signalling pathways influencing gene transcription or enzyme activity (Dikalova et al. 2010; Sachse & Wolf 2007; Zhang et al. 2007; Spät & Hunyady 2004; Hunyady & Catt 2006). However, there is evidence to suggest AngII may also act directly on mitochondria.

1.11. Mitochondrial RAS

The evidence to support a RAS operating within or in close association with mitochondria is gathered from studies spanning the last 40 years. The first evidence came from studies using radiolabelled AngII to identify intracellular sites of action. Exogenously administered ^{3}H-labelled AngII trafficked to the surface of rodent cardiac mitochondria (Robertson & Khairallah 1971) whilst radioiodinated AngII (^{125}I-AngII) bound to the mitochondrial fraction of rat neural tissue (N E Sirett et al. 1977). Since then, AngII binding has been demonstrated in mitochondria from kidney and adrenal tissue (van Kats et al. 2001) whilst AngII and AT_1R have been reported to co-localise on the outer mitochondrial membrane in rat vagal sensory cortex (Huang et al. 2003) and cerebellar cortex (Erdmann et al. 1996).

Rather than cytosolic AngII operating at the outer mitochondrial membrane, there is also evidence to suggest that an intra-mitochondrial RAS might exist, capable of *de-novo* AngII synthesis. Intra-mitochondrial 'dense bodies' isolated from the rat adrenal cortex were reported to contain renin, angiotensinogen and ACE (Peters et al. 1996; Rong et al. 1994) whilst renin co-localises with mitochondria on confocal microscopy of cardiomyoblasts (Wanka et al. 2009). The intramitochondrial presence of AngII precursors and necessary AngII forming enzymes would argue for the existence of an intramitochondrial RAS (mRAS).

Any such mRAS could not be wholly mitochondrial since both renin and angiotensinogen are nuclear encoded proteins. Their presence within mitochondria must be dependent on either protein or RNA transfer across mitochondrial membranes. Both these processes are well recognised. Human mitochondria are able to import both mRNA (Ahmed & Fisher 2009) and transfer RNA (tRNA) (Rubio et al. 2008) into the matrix where they are translated by ribosomes associated with the inner mitochondrial

membrane (Liu & Spremulli 2000) and inserted into the inner membrane by the oxidase assembly machinery (Herrmann & Neupert 2003; Schmidt et al. 2010). In support of such mechanisms enabling the construction of an mRAS, a truncated renin mRNA transcript (exon-1A renin) lacking the secretory pathway signalling sequence has been identified in adrenal gland tissue which seems able to be transported across the mitochondrial membranes (Clausmeyer et al. 2000; Clausmeyer et al. 1999).

However, it is not necessary to prove the existence of truncated mRNA isoforms of all necessary RAS proteins; not all mitochondrial proteins are translated within mitochondria. The vast majority of mitochondrial proteins are synthesised in the cytosol and imported into one of the four mitochondrial compartments; the outer membrane (Endo & Yamano 2010), intermembrane space (van der Laan et al. 2010), inner membrane (Herrmann & Neupert 2003) or mitochondrial matrix (Wagner et al. 2009). As a result, whilst the mitochondrial genome encodes only 13 proteins, upwards of 1500 proteins have been identified in the human mitochondrial proteome (Lopez et al. 2000).

Taken together, these data suggest that a mitochondrial RAS may exist, with component proteins and enzymes variously imported intact or as mRNA precursors.

1.12. Hypothesis

The introduction has described the evidence for the influence of the RAS on mitochondrial function and data supporting the existence of a mitochondrial RAS. However, these data are gathered from studies in a range of tissues with no work identifying a complete intramitochondrial system.

I therefore propose the following hypothesis:

H1: There is a local renin-angiotensin system in liver mitochondria.

I sought to examine this hypothesis in liver since this is a tissue with a high mitochondrial content consisting of a single homogenous population. Other tissues such as muscle have multiple mitchondrial populations (in muscle; subsarcolemmal and intermyofibrillar) which are distinct in activity and function. Separating these populations is scientifically necessary, but reduces the yield considerably. Organs such as kidney are smaller in size and contain more connective tissue, making homogenization difficult, again reducing the yield. Liver, being a large organ with little connective tissue and a high mitochondrial density, provides an aqaquate yield to support the experiments conducted in this work. Further, a mitochondrial RAS is likely to be highly conserved in evolutionary terms given mitochondrial phylogeny; it is likely to be ubiquitous and as such should be present in liver tissue.

Four complementary techniques were employed to ascertain whether RAS proteins exist within mitochondria and whether a functional effect of such a RAS could be identified. Proteomic surveys of both existing databases and isolated liver mitochondria were conducted, alongside attempted identification of AngII receptors by immunoprecipitation and Western blotting. Radioligand binding studies using labelled AngII were performed with mitochondrial and associated membranes and respirometric

studies with isolated crude mitochondria were run. Chapter 2 describes the methods used in all these experiments.

In Chapter 3 I show that no RAS proteins could be identified in liver mitochondria by proteomic survey. In Chapter 4 I describe attempts to identify AngII receptors, which were ultimately unsuccessful. Chapter 5 goes on to show that AngII binds to MAM but not to pure mitochondria, and that the binding sites are likely AT_1R. Finally, Chapter 6 details the limited effect of AngII on the respiration of isolated mitochondria.

1.13. Background data from within research group

(Work conducted by Dr James Skipworth)

1.13.1. Lack of detectable ACE in liver mitochondria

If a fully functional intramitochondrial RAS exists in liver mitochondria, enzymes catalysing the final essential step of AngII synthesis from AngI should be present. As the most evolutionary conserved of all the RAS components, ACE is the most likely candidate to perform this function especially given its apparent presence in intramitochondrial dense bodies in the zona glomerulosa (Peters et al. 1996). The presence of ACE was thus sought in purified mitochondria from rat liver. An antibody directed to the C-terminus of the protein recognised a high molecular weight (MW) band at the predicted weight of ACE (MW ≈ 150 KDa) in the homogenate, nuclear, lysosomal and microsomal fractions but not in the mitochondria. However, a low MW band (≈ 50 kDa) band was enriched in the crude mitochondrial and pure mitochondrial fractions.

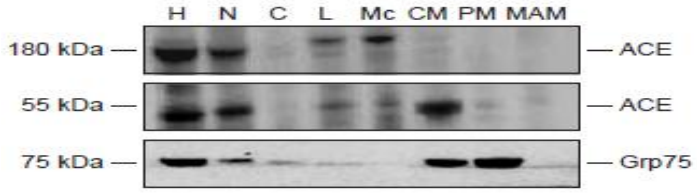

Figure 5. Western blot using anti-ACE and anit-grp-75 antibodies in rat liver subfractions. H-homogenate, N-nuclear fraction, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation (CM) and further separated into mitochondria associated membranes (MAM) and pure mitochondrial (PM) fractions by isopicnic Percoll centrifugation (for details see Methods 2.2). Mitochondrial fractions contain only a non-canonical 50kD immunoreactive band.

Since a shorter isoform of human and rat ACE exists (ACE-T, Uniprot P47820) the identity of the lower MW band was sought by mass spectrometry of the immunoprecipitated protein. However, analysis of this band did not return any ACE related sequences, indicating non-specific binding by the antibody.

Whilst these data appeared to exclude the possibility of intramitochondrial generation of AngII by ACE, they did not exclude AngII generation by other enzymes. Further, rather than being generated within the mitochondrial matrix, AngII may be imported into mitochondria or act upon the outer membrane.

I conducted a series of experiments to investigate these possibilities.

2. METHODS

2.1. Mass Spectrometry

2.1.1. Introduction

Mass spectrometry is a powerful analytical tool used to measure the anatomical mass of a given sample. For biological samples such as peptides, it can measure to an accuracy of 0.01%, allowing for the differentiation of proteins according to amino acid substitutions or post translational modifications.

Modern mass spectrometers consist of three main components; an ionisation source, an analyser and a detector. The principle of the technique depends on sample molecules being ionised, which then allows them to be separated according to their mass (m)-to-charge (z) ratios (m/z). Once separated, the detector produces a signal combining the m/z ratio value, the molecular mass and relative abundance or each ion (m/z spectrum).

There are various methods for sample ionization, though the most frequently employed are Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption Ionisation (MALDI). In ESI, the sample is dissolved in a polar, volatile solvent which is then sprayed through a fine capillary tube to which a high voltage is applied. The emerging droplets become highly charged and as they evaporate produce individual charged sample ions free from solvent. In MALDI, sample molecules are bombarded with laser light to bring about ionisation. To avoid decomposition of the analyte by direct expose to laser energy, the sample is pre-mixed with a matrix compound which absorbs laser energy, transforming it into excitation energy for the sample.

2.1.2. Peptide sequencing by Mass Spectrometry

To sequence peptides by mass spectrometry, first the large peptide molecules must be broken into constituent fragments. For peptide sequencing, digestion of the sample

using sequence specific cleavage agents, such as trypsin, is most often employed. The digested mixture is analysed using ESI or MALDI without further separation in the first instance to generate a list of molecular masses.

From this mix, individual peptides are selected to be analysed further by tandem mass spectrometric amino acid sequencing to generate sequence information. In this, selected sample ions are passed into a collision cell and bombarded by gas molecules (usually argon) to cause fragmentation. Fragmentation occurs at reasonably well documented points (Roepstorff & Fohlman 1984) with the number of fragments determined by the energy of the collider; time-of-flight colliders cause less fragmentation. These fragments are then analysed by a second analyser to provide m/z ratios (Phillips et al. 2012).

The amount of sequence information varies from one peptide to another. Whilst some proteins provide enough information to cover 70-80% of a protein sequence, often a sequence of 4-5 amino acids from a single proteolytic peptide product is sufficient to identify a protein from a database.

The mass spectrometry assays included in this thesis were performed by experienced technical staff in the Taplin Mass Spectrometry facility at Harvard Medical School (MA, USA) using a dual source (MALDI and ESI) time of flight mass spectrometer (Orbitrap mass spectrometers,Thermo Scientific, MA, USA).

2.2. Isolation of mitochondria and other subcellular fractions

2.2.1. Isolation of subcellular fractions

Livers from 12-week old male Sprague-Dawley rats were excised and placed in ice cold *isolation medium A* (see Appendix 1.1) before mincing and washing in the same medium, supplemented with a protease inhibitor cocktail. Livers were homogenised in a Potter-Elvehjem homogeniser, and the homogenate centrifuged at 800 g for 10 minutes. Nuclear material formed the pellet with a post-nuclear supernatant. The supernatant was centrifuged at 10,300 g for 10 minutes resulting in the crude mitochondrial (CM) pellet and post-mitochondrial supernatant. This supernatant was then centrifuged at 25,000 g for 30 minutes producing a lysosomal pellet. All these steps were performed using a Beckman J2-MC Centrifuge with a JA-20 rotor. Finally, the post lysosomal 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 A* in a cryovial before snap freezing in liquid nitrogen and storing at -80 °C. All isolation steps, including centrifugation, were carried out at 4 °C.

2.2.2. Isolation of pure mitochondria and mitochondria assocaited membranes

Percoll density gradient separation was used to separate pure mitochondria (PM) from mitochondria assocaited membranes (MAM). Crude mitochondria were prepared as described above and then layered on a column of 30% (vol/vol) Percoll in *isolation medium B (Appendix 1.1)* 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, see Fig 6C) were aspirated and diluted in 500 µl of *isolation medium A* before being centrifuged at 6300 g for 10 minutes (Beckman J2-MC Centrifuge; JA20.1 rotor). The pellet of the PM fraction was resuspended in 500 µl of *isolation medium A* in

a cryovial and snap frozen in liquid nitrogen. The supernatant from the MAM fraction was then subjected to centrifugation at 100,000 g for 1 hour (Beckman L-70 Ultra-Centrifuge with 70.1 Ti rotor) to pellet the purified MAM. The pellet was resuspended in 500 µl of *isolation medium A* (without protease inhibitor) in a cryovial, snap frozen in liquid nitrogen and stored at -80 °C.

2.2.3. Determination of mitochondrial protein topology

Proteinase K digestion was used to differentiate proteins integral to the mitochondrial outer membrane from those residing in the inner membrane and matrix. When isolated mitochondria are exposed to Proteinase K, proteins expressed on the outer membrane will be cleaved but the outer membrane remains intact, protecting inner membrane proteins from digestion. Sodium carbonate extraction can then be performed, lysing mitochondria and thus freeing matrix proteins. The inner membrane can then be isolated and the matrix proteins precipitated.

Proteinase K was added to 50 µg of PM to a final concentration of 50 µg/ml and incubated on ice for 10 minutes. Phenylmethanesulfonyl fluoride (PMSF) was then added to a final concentration of 1 mM to inhibit Proteinase K. Following a further 10 minute incubation on ice the mitochondria were pelleted by centrifugation at 10,000 g for 10 minutes (Beckman J2-MC Centrifuge with a JA-20 rotor), resuspended in 50 µl *isolation medium A* and a sample (PK) snap frozen in liquid nitrogen and stored at -80 °C.

To lyse mitochondria, 50 µg of proteinase K digested PM were resuspended in 100 µl sodium carbonate solution and incubated on ice for 30 minutes. Mitochondrial membranes were pelleted by centrifugation at 100,000g for 30 minutes (Beckman L-70 Ultra-Centrifuge with 70.1 Ti rotor). The soluble proteins in the supernatant were then

precipitated using 72 % Trichloroacetic acid (TCA). This sample represents the matrix proteins (MX).

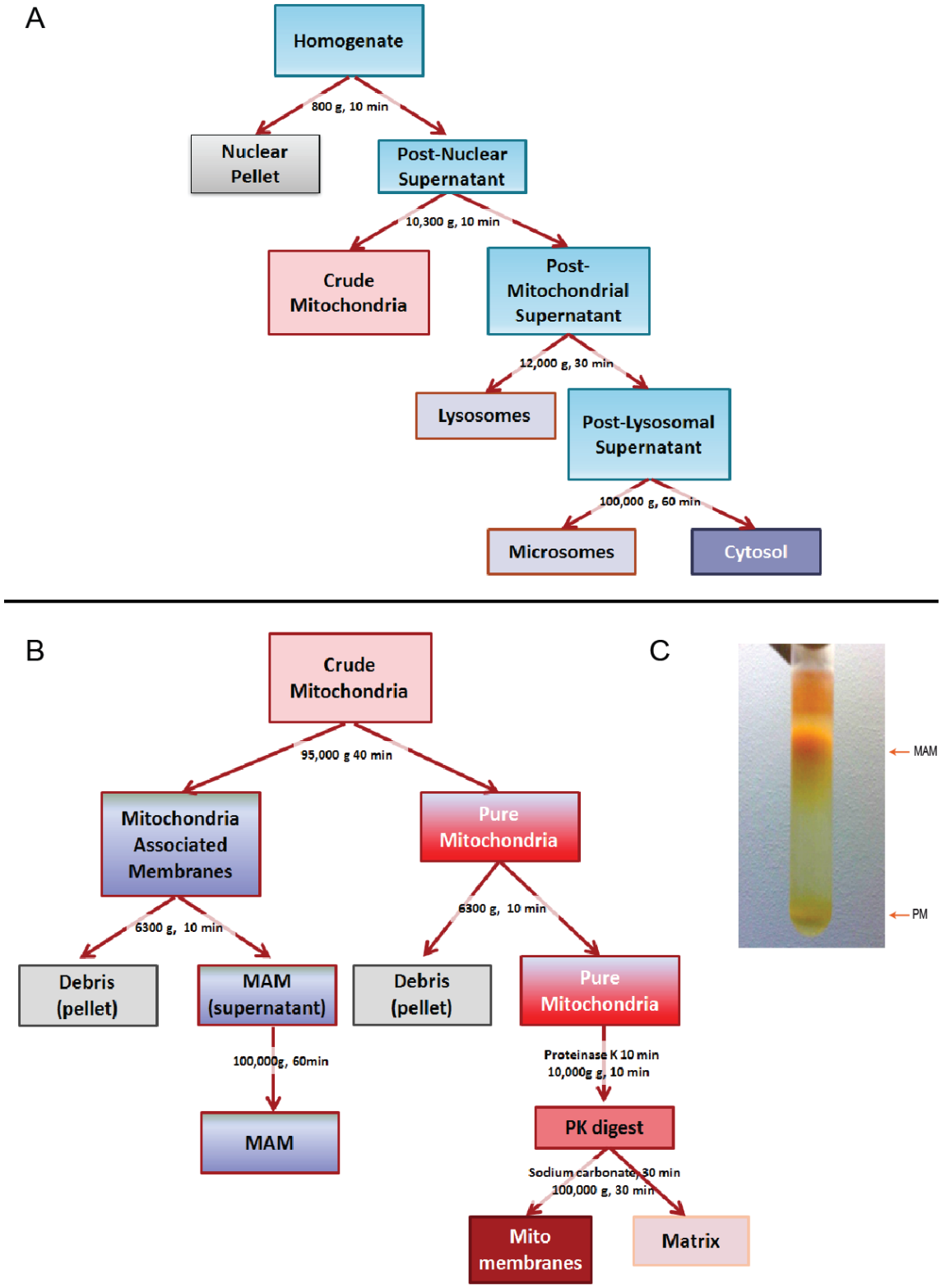

Figure 6. Protocol for isolation of subcellular fractions and mitochondrial membranes. (A) Sequential centrifugation to isolate subcellular fractions. (B) Ultracentrifugation protocol for crude mitochondrial separation. (C) Photograph of centrifuge tube following ultracentrifugation of crude mitochondrial fraction. MAM comprise the top band and pure mitochondria (PM) the lower band.

2.2.4. TCA precipitation of proteins

Protein samples were diluted with 72 % TCA to make a final 12 % TCA concentration and incubated on ice for 30 minutes. Samples were then centrifuged at 18,000 g for 30 minutes at 4 °C. The pellet was washed in 200 µl of acetone and centrifuged at 10,000 g for 10 minutes. This acetone wash was repeated twice. The resulting washed pellet was allowed to dry at 95 °C for 10 minutes and then resuspended in 50 µl *isolation medium A*, snap frozen in liquid nitrogen and stored at -80 °C.

2.2.5. Protein quantification

The protein concentration of a given sample was determined using bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. This method utilises the reducing property of proteins in an alkaline solution to reduce Cu^{2+} to Cu^{1+}. The chelation of two molecules of BCA with one Cu^{1+} produces a purple colouration of the solution, which exhibits a strong linear absorbance at 562 nm as concentration increases. The absorbance of a sample can then be compared to that of a known set of standards (diluted over the working range of 20 – 2000 µl/mL).

Samples were lysed by mixing 1:1 with non-BSA containing lysis buffer (see Appendix 1.3) and incubating for 10 minutes. DNA was pelleted by centrifugation at 16,100 g for 10 minutes and the supernatant removed for quantification. Each sample was assayed in duplicate on a 96 well plate with 5 µl of protein sample added to each well. A series of known protein concentrations was prepared from an albumin standard (2 mg/mL) and 5 µl of each added to wells of the plate in duplicate. 195 µl of BCA reagent was added to each well and the plate then incubated at 37 °C for 30 minutes. Absorbance was measured at 562 nm using a spectrophotometer. The absorbance of the blank standard was subtracted from all other measurements. A standard curve was generated from the absorbance of the known standards and a trend line fitted. The protein concentration of the samples was calculated according to the equation of the trend line.

2.3. Western blotting

2.3.1. Introduction

Western blotting is used to detect specific proteins by way of separation according to size. Gel electrophoresis can separate native proteins based on their 3D structure or denatured proteins by the length of the polypeptide. The gels are usually polyacrylamide (made from acrylamide and N,N-methylenebisacrylamide (Bis)) and are neural, hydrophilic, 3D networks of long hydrocarbons cross-linked by methylene groups. The size of the pores in this 3D matrix is determined by the relative amount of Bis and acrylamide, giving the gels a % w/v property. Sodium dodecyl sulfate (SDS) added to protein samples gives each protein a negative charge proportional to its mass. Passing a voltage through the gel causes the negatively charged proteins to migrate away from the anode according to their charge and therefore mass.

Once proteins are separated within the gel, they can be induced to transfer onto a membrane by application of an electric field. The gel is stacked on top of a membrane, and filter paper placed either side. The stack is placed with the membrane closest to the positive electrode so that the negatively charged proteins migrate from gel to membrane.

2.3.2. Protocol

Samples were diluted 1:4 with Laemmli buffer (Appendix 1.3) and incubated in boiling water for 10 minutes to denature proteins. Proteins were resolved using 4-12% Bis-Tris glycine pre-cast gels (Invitrogen, Paisley, Uk). Samples were prepared with 5 x SDS buffer (1:5) before heating in boiling water for 10 mins. Equal amounts of protein were loaded into wells alongside a molecular marker cocktail. Gels were run at 100 V in 1 x SDS running buffer (Appendix 1.3). Once the dye had migrated through the gel,

proteins were transferred from the gel to nitrocellulose or polyvinylidene diflouride (PVDF) membranes using a semi-dry transblot electro-transfer system (Bio-Rad, CA, USA). Membranes were activated in 100% methanol for 1 minute before being wetted in transfer buffer (Appendix 1.3), layered beneath the gel and run for 1 hour at 20 V. Once the transfer was complete membranes were blocked for 1 hour with 5% bovine serum albumin (BSA) diluted in phosphate-buffered saline with Tween-20 (PBST) (Appendix 1.3) or alternatively PBST with 5% fat-free milk. Membranes were then washed 3 times in PBST before incubation with primary antibody.

Following primary incubation, membranes were washed three times for 5 minutes in PBST before incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Appendix 1.3). Membranes were developed with the enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

2.3.3. Coomassie staining

Proteins were visualised in the gel by treating them with a solution of 7 % acetic acid, 40 % ethanol and 0.1 % Coomassie Brilliant Blue (Life Sciences, UK). Proteins are visualised as blue bands on a clear background. Following imaging, excess stain was removed by multiple washes in a solution containing 0.7 % acetic acid and 20 % ethanol.

2.4. Validation of mitochondrial membrane isolation

In order to validate the mitochondrial isolation protocol, Western blots were performed with all cell subfractions. Membranes were probed with antibody to CoxIV (#4844, Cell Signalling, MA, USA), an integral inner mitochondrial membrane protein of 17 KDa.

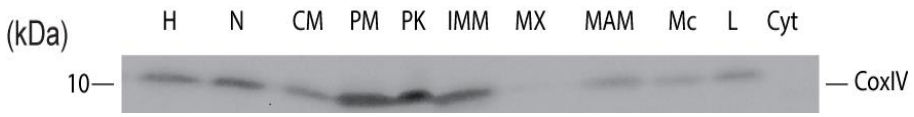

Figure 7. Cox IV immunostaining of subcellular fractions. Following the protocol of differential centrifugation, nuclear (N) microsomal (Mc), Lysosomal (L), cytosolic (Cyt) and crude mitochondrial (CM) fractions were isolated. Percoll density ultracentrifugation of the CM fraction separated pure mitochondria (PM) from mitochondrial-associated membranes (MAM). Proteinase K was used to digest outer membrane proteins (PK) before a sodium carbonate extraction of the PK fraction separated inner mitochondrial membranes (IMM) and mitochondrial matrix (Mx) proteins. Each lane of a Bis-Tris 4-12% agarose gel was loaded with 40 µg of protein. Proteins were transferred onto a PVDF membrane before incubation with primary antibody for 16hrs at 4 ˚C followed by application of a horseradish peroxidase conjugated secondary antibody for 2 hours. Cox IV immunoblotting revealed distinct bands in H, N, CM PM, PK and IMM, with faint bands in MAM, Mc, L and Cyt.

The results with CoxIV immunoblotting suggest that the isolation technique successfully enriches mitochondria in the pure mitochondrial fraction. The pattern of increasingly intense bands in the crude and pure mitochondrial fractions is in keeping with an integral mitochondrial protein. The lack of change in intensity following Proteinase K digestion is consistent with the inner mitochondrial membrane location of CoxIV, as well as suggesting maintained inner mitochondrial membrane integrity. Faint bands in other fractions suggest minor mitochondrial contamination.

2.5. Immunoprecipitation

2.5.1. Introduction

Immunoprecipitation refers to the process of antigen affinity purification utilising a specific antibody. When incubated with the antigen-containing solution, antibody and antigen will form a complex. By immobilising the specific antibody by attachment to an insoluble support of high molecular weight (such as an agarose bead) the complex it forms with the specific antigen can be separated from a complex solution (such as cell lysate) by centrifugation.

Protein A, Protein G and Protein A/G are the most commonly employed immunoglobulin-binding proteins and each shows specificity for the heavy chains on the Fc region of antibodies. When immobilised on an agarose bead they are effective tools for attaching antibody.

Since the antibody binding affinity of Protein A/G is non-specific (and will thus bind any other antibody in solution), a pre-clearing step is usually employed to clear the solution of non-specific antibody. Protein A/G is incubated with sample solution and then subjected to centrifugation; the pelleted Protein A/G will be bound to non-specific antibodies. The supernatant can then be used as the input for the immunoprecipitation step with the specific antibody of choice.

2.5.2. Protocol

Samples were solubilised using immunoprecipitation buffer (containing Triton X-100, protease inhibitor and phosphatase inhibitor) and lysates were centrifuged at 14,000 rpm for 30 minutes. The supernatants (400 µg of total protein) were first pre-cleared with equilibrated Protein A/G sepharose beads (GE Healthcare, Buckinghamshire, UK). Twenty µl of Protein A/G was added to 400 µg of sample proteins and rotated in tubes

for 1 hour. Following centrifugation, the supernatant (input) was incubated overnight with Protein A/G and 2 µg of primary antibody (Sc-1173; anti AT_1R, or sc 9140; anti-AT_2R, both Santa Cruz, CA, USA). Further centrifugation separated the antigen bound bead complex (pellet) from the output (supernatant). The pellet was resuspended in sample buffer before heating at 95 °C to separate antigen from antibody-Protein A/G complex. After a further centrifugation, the antigen of interest along with antibody devoid of agarose bead- protein A/G complex (elute) forms the supernatant. Immunoprecipitated proteins were resolved using NuPAGE Bis-Tris gels (Invitrogen, Paisley, UK) as described previously.

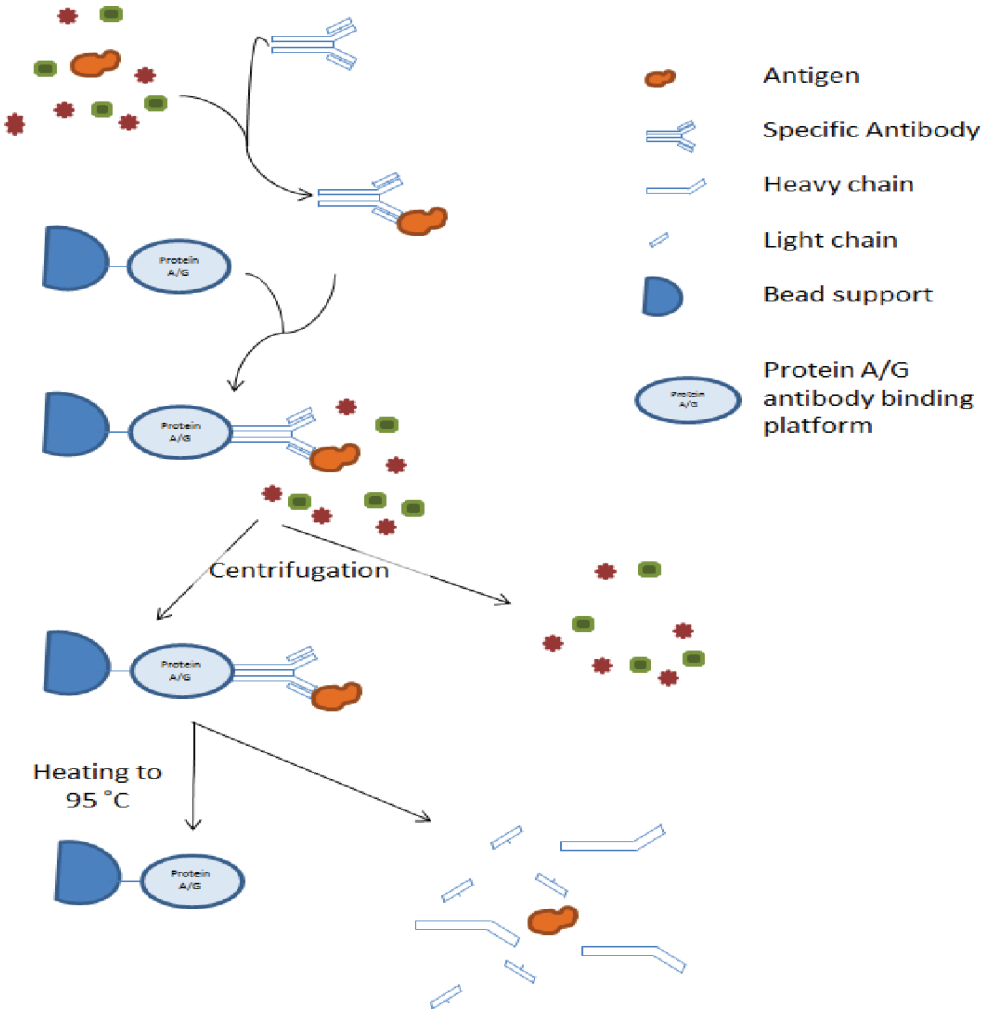

Figure 8. Schematic of immunoprecipitation using Protein A/G and agarose beads.

2.6. Cell culture and small interfering RNA inoculation

2.6.1. HepG2 cell culture

HepG2 cells are a perpetual human cell line derived from a well-differentiated hepatocellular carcinoma in a Caucasian male. They are epithelial in morphology and display a high degree of functional differentiation *in vitro* making them a suitable model for studies of liver metabolism. HepG2 cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlbad, CA, USA) supplemented with foetal bovine serum and streptomycin. Flasks were incubated at 37˚C in 5% CO_2 atmosphere.

2.6.2. Small interfering RNA inoculation

HepG2 cells were cultured in 6 well plates (at approximately 250,000 seeded cells/well on day 0). On day 1 (at approximately 60% confluency), cells were transfected with the chosen small interfering RNA (siRNA). Two vials were prepared; first 10 µl of siRNA was added to 190 µl DMEM, secondly 6 µl DharmaFECT 4 (GE Healthcare, Amersham, Herts, UK) was added to 194 µl DMEM. After 5 minutes, the vials were mixed and left at room temperature for 20 minutes. The media in the 6 well plates was removed and 1.6 ml DMEM added. The siRNA mix was then added and the cells incubated for 48hours at 37 ˚C in 5% CO_2 atmosphere. Harvesting of cells was achieved with 0.25% Trypsin (#T4049 Sigma Aldrich, Dorset, UK) and centrifugation (3000 rpm, 4 minutes). Pelleted cells were stored at -80 ˚C. Cell lysates were prepared using non-BSA containing lysis buffer, supplemented with protease and phosphatase inhibitors.

2.7. Radioligand binding

2.7.1. Introduction

Ligand binding assays can measure both the affinity of ligands for a given receptor and also the density of that receptor in a membrane preparation. Three variables are of particular interest; the equilibrium dissociation constant (K_D), the maximum density of receptors (B_{MAX}) and the Hill coefficient (nH). The K_D denotes the concentration of ligand that will occupy 50% of the receptors and as such is a measure of strength of ligand-receptor interaction. B_{MAX}. is measured in amount of ligand/mg protein assayed. Saturation binding assays can be used to determine both these variables. The Hill coefficient describes the binding cooperativity of a receptor site.

2.7.1.1. Saturation binding

Saturation binding refers to a technique in which membranes are exposed to radiolabelled ligand in optimal binding conditions and the amount of bound ligand at equilibrium quantified. This is achieved by incubating membrane preparations with radiolabelled ligand for a pre-determined time (and temperature) to reach equilibrium, which is then broken by centrifugation. Unbound ligand can be removed from bound ligand by resuspension in Tris-HCl and re-centrifugation. Unbound ligand will be retained in the supernatant, whilst bound labelled ligand can be measured by Geiger counter. However, binding of ligand will include both binding to specific binding sites and non-specific binding.

Non-specific binding can be quantified by running experiments in parallel in which membrane preparations are incubated with radiolabelled ligand and 1000 fold excess of unlabelled ligand. The excess unlabelled ligand will compete successfully for specific binding sites leaving radioligand to bind non-specific sites only. Once quantified (as

before) this value for non-specific binding can be subtracted from the total binding value to give specific binding. Plots of these binding values can give an approximation of B_{MAX} (the amount of specific binding observed where the isotherm plateaus) and the K_D (the ligand concentration at 50% of B_{MAX}) (Fig. 9). The shape of these curves roughly describes the Hill slope. A value of 1.0 denotes a ligand binding to a single site with no cooperativity (that is, binding of ligand does not increase the affinity of the receptor to bind further ligand). A value greater than 1.0 suggests more than one binding site with positive cooperativity; less than 1.0 suggests multiple binding sites with varying affinity or negative cooperativity. More accurate estimates can be made from linearising the data in a Scatchard plot. More commonly, complex line fitting programmes are employed. In the work presented, data were analysed using the iterative non-linear curve fitting programmes Kinetic and LIGAND (KELL package, Elsevier Biosoft, Cambridge, UK).

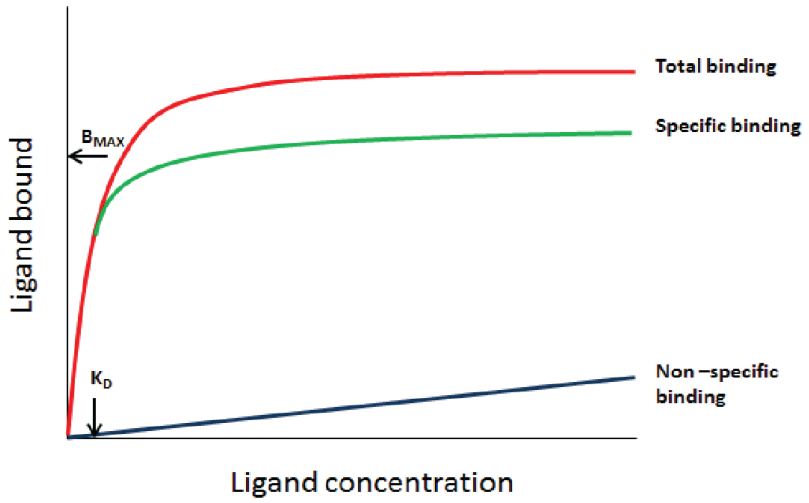

Figure 9. Graphical representation of a ligand-binding saturation assay. Total binding is measured with radiolabelled ligand alone, giving a saturation curve (in red). Non-specific binding is measured with radiolabelled ligand in the presence of 1000 fold excess unlabelled ligand. Labelled ligand will bind non-specific sites in a linear fashion (blue line). Specific binding can then be calculated by deducting non-specific from total binding values.

2.7.1.2. Competition binding

With the K_D defined for a given ligand in saturation assays, the ability of other ligands to displace this specific binding can be measured. Competition curves are obtained by plotting specific binding in the presence of competitors as a percentage of total binding, against the concentration of the competing ligand. With reference to AngII receptors, the specific inhibitors of AT_1R (losartan) and AT_2R (PD123319) were used to assess receptor type.

2.7.2. Protocol

2.7.2.1. Saturation binding

Mitochondrial homogenates (100 µL) were incubated in assay buffer (Appendix 1.4) with increasing concentrations (15 pM-2 nM) of [^{125}I]-AngII (in a final volume of 200 µL) for 1 hour at room temperature (22 ˚C). Unlabeled AngII (10 µM) was used to determine non-specific binding at each concentration in duplicate tubes. Homogenates were centrifuged (20,000 g, 10min, 4 ˚C) to break equilibrium, pellets re-suspended in 500 µL ice-cold Tris-HCL (50 mM, pH 7.4) 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 K_D, B_{MAX} and nH.

2.7.2.2. Fixed concentration competition assays

Homogenates (100 µL) were incubated in assay buffer as previously with 0.5 nM [^{125}I]-AngII (in a final volume of 200 µL) for 1 hour at room temperature (22 ˚C), with or without the addition of AngII (10 µM) to determine non-specific binding. Additionally,

either the AT_1R specific antagonist losartan (1 µM) or the specific AT_2R antagonist PD123319 (1 µM) were included to determine presence of AT_1R vs. AT_2R.

2.8. Respirometry

2.8.1. Introduction

Since the reduction of O_2 at complex IV is a crucial step in OXPHOS, measuring oxygen consumption of tissues, cells or isolated mitochondria has been a cornerstone in investigations of mitochondrial (dys)function since it was first developed by Chance and Williams in the 1950s. Since then, various apparatus have been used in the pursuit of increasingly accurate measurements of mitochondrial oxygen consumption. However, the function of the majority of such apparatus is based on the same principals governing the early Clark electrodes.

The Clark-type polarographic oxygen sensor consists of a gold cathode, a silver/silver chloride anode and a potassium chloride electrolyte reservoir. The sensor is separated from a sample suspended in solution within a chamber by a 25 µM membrane, across which a fixed voltage is applied (usually 0.8 V). Oxygen diffuses from the sample solution to the cathode surface across the membrane and then the electrolyte layer. As oxygen diffuses across the membrane it is reduced by the fixed voltage which then generates a current. Since the oxygen tension is held at close to zero at the cathode, under steady-state conditions the oxygen flux to the cathode depends on the external oxygen pressure and is therefore in proportion to the concentration of oxygen in the solution.

The raw signal is converted to oxygen concentration (µM or $nmol.ml^{-1}$) by calibration of the oxygen sensor against two-points; usually achieved at air saturation and zero oxygen concentration. The measured oxygen flow can then be converted to an oxygen flux per sample size (e.g. mass of mitochondrial protein).

Various factors can affect the sensitivity of a Clark electrode. In a 2 ml volume of liquid, oxygen concentration will vary according to distance from the air/surface interface. This can result in the membrane being exposed to an unrepresentative sample of fluid. To minimize this, a stirrer is used to effectively mix the sample throughout the course of the experiment. The permeability of the membrane will also affect the signal at the oxygen sensors and for this reason it must be kept at constant temperature. Residual oxygen diffusion from stir bars, stoppers and from the chamber materials themselves can all affect flux readings.

The Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria) is a high resolution system which combines precise temperature control with a system built with inert materials and sensitive membranes resulting in a residual oxygen consumption of less than 2 $pmol.s^{-1}.ml^{-1}$ and a detection limit of 0.5 $pmol.s^{-1}.ml^{-1}$, allowing accurate measurement of oxygen flux in small amounts of tissue. Whilst measurements can be made in whole cells, the permeability of the cell membrane means that some mitochondrial modulators are not effective. A more detailed analysis of mitochondrial pathways can be made with isolated mitochondria.

2.8.2. Mitochondrial isolation for respirometry

Liver mitochondria were isolated immediately after tissue harvesting at $4^{o}C$. The excised livers were immersed in ice-cold *isolation medium A* (Appendix 1.1) and minced with scissors followed by homogenisation by Potter-Elvehjem homogeniser. The homogenate was centrifuged for 10 minutes at 700 g. The supernatant was collected and centrifuged three times for 10 minutes at 7,000 g. Pellets (CM) were re-suspended in *isolation medium A* to a final concentration of 50-100 mg protein ml^{-1}. Protein concentration was measured using the Pierce BCA Protein Assay kit (Thermo scientific, MA, USA) according to manufacturer instructions.

2.8.3. Experimental conditions

All experiments were conducted at 37 ˚C to replicate *in vivo* conditions. However, oxygen concentrations over the course of the experiment are non-physiological. *In vivo*, oxygen partial pressure (pO_2) decreases as it cascades from the atmosphere (20 kPa) to alveolar (13 kPa), mixed capillary (3 kPa) and sarcoplasmic (0.3-0.5 kPa) environments. This intracellular pO_2 equates to roughly 3 µM or 3 nmol ml^{-1} (Gnaiger et al. 1998). However, by virtue of the design of oxygen consumption experiments, the starting pO_2 needs to be greater than this in order for O_2 availability not to be rate limiting during the course of the experiment. Conversely, exposing isolated mitochondria to supra-physiological pO_2 may induce oxidative stress through excess ROS production or be directly toxic. This dilemma is well recognised in the field of mitochondrial respirometry. As a practical compromise, experimental oxygen concentrations were maintained in the range 200 - 50 nmol/L (200 - 50 µM) ensuring that respiration was not restricted by O_2 availability. Additionally, since all experiments were run with contemporary control assays, all mitochondrial samples were exposed to the same conditions.

2.8.4. Measurement of mitochondrial respiration

Using the Clark electrode, various aspects of mitochondrial respiration can be interrogated. State 2, State 3 and State 4 respiration can all be measured by using a chosen substrate and measuring respiration rates before addition of ADP (State 2), respiration on ADP (State 3) and respiration following consumption of ADP (State 4). However, this measure of State 4 is susceptible to inaccuracies caused by contamination of the sample with ATPase (recycling ADP). A more accurate measure of State 4 respiration can be achieved by using an ‘uncoupler’ of the ETS such as oligomycin (which is an inhibitor of ATP synthase). This effectively inhibits complex IV

and thus gives an accurate measure of non-ETS coupled or LEAK respiration, termed State 4_{oligo}.

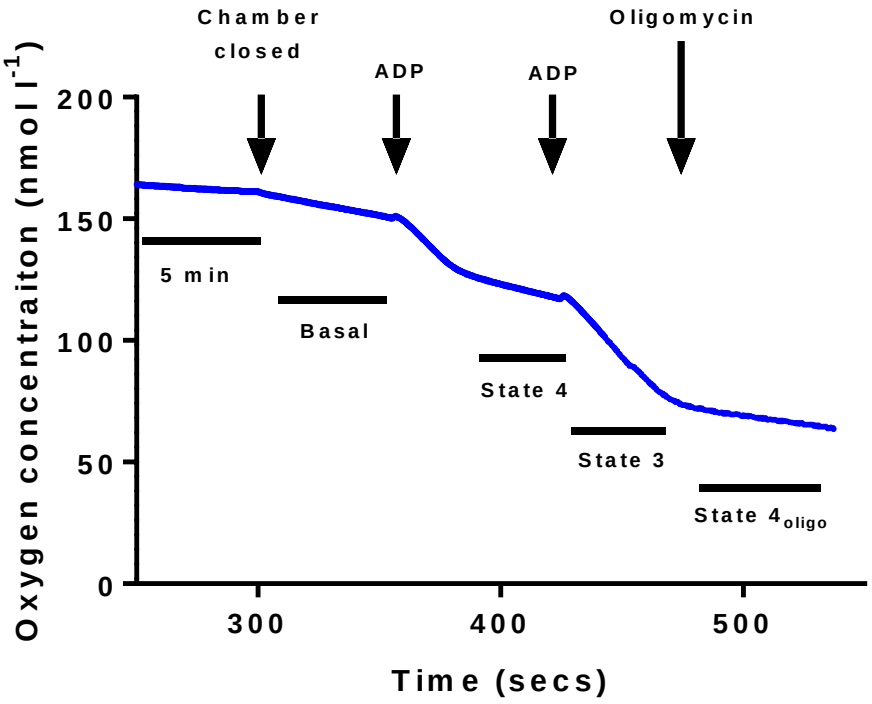

Figure 10. Representative trace of respirometry assay using Hansatech oxygraph. Mitochondria were suspended in respiration medium (Appendix 1.5) with pyruvate (5 mM) and malate (5 mM) and allowed to respire for 5 minutes in the absence of exogenous ADP. ADP was added in a submaximal dose to allow for State 4 measurement. State 3 was measured in the presence of a saturating concentration of ADP. LEAK respiration was assessed after adding oligomycin.

2.8.4.1. Individual Complex activities

The activity of individual complexes of the ETS can be assessed by using substrates specific to that complex in the presence/absence of inhibitors of other complexes (Fig. 11). Glutamate and pyruvate, in the presence of malate (but not malate on its own) support complex I respiration by providing NADH via the TCA cycle. Complex II activity can be isolated by applying the complex I specific inhibitor rotenone and the addition of succinate (the substrate for the succinate dehydrogenase subunit of complex II). Similarly, complex IV activity can be isolated by providing its (artificial) substrates N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and ascorbate, in the presence of antimycin A (a specific inhibitor of complex III). Due to active auto-oxidation of reduced TMPD in the chamber, sodium azide is often added to terminate State 3 respiration at complex IV. In this way, pathology relating to individual complexes can be identified.

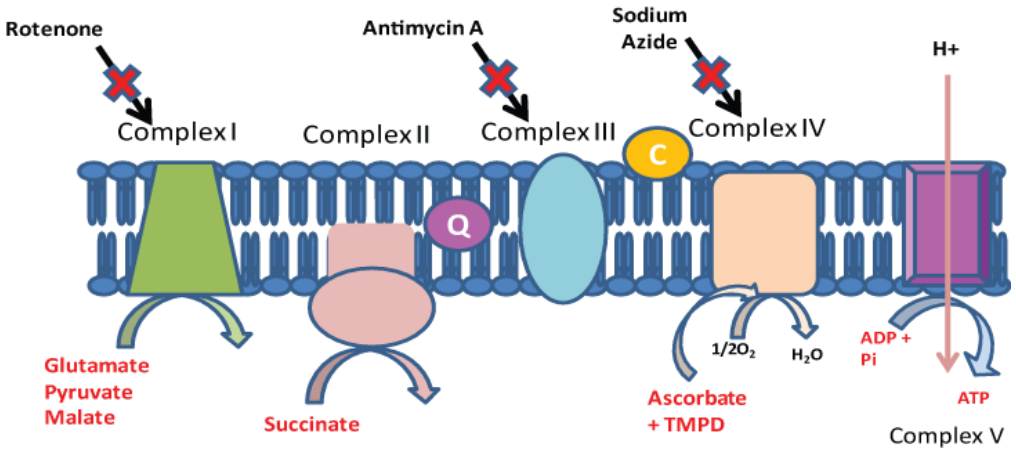

Figure 11. Substrate-inhibitor titration for respirometry in isolated mitochondria. Glutamate, pyruvate and malate support complex I respiration. Addition of rotenone inhibits complex I allowing complex II respiration to be assessed with addition of its substrate, succinate. Complex IV respiration is stimulated by addition of ascorbate and TMPD having inhibited complex III with antimycin A. Q; Coenzyme Q, C; Cytochrome C.

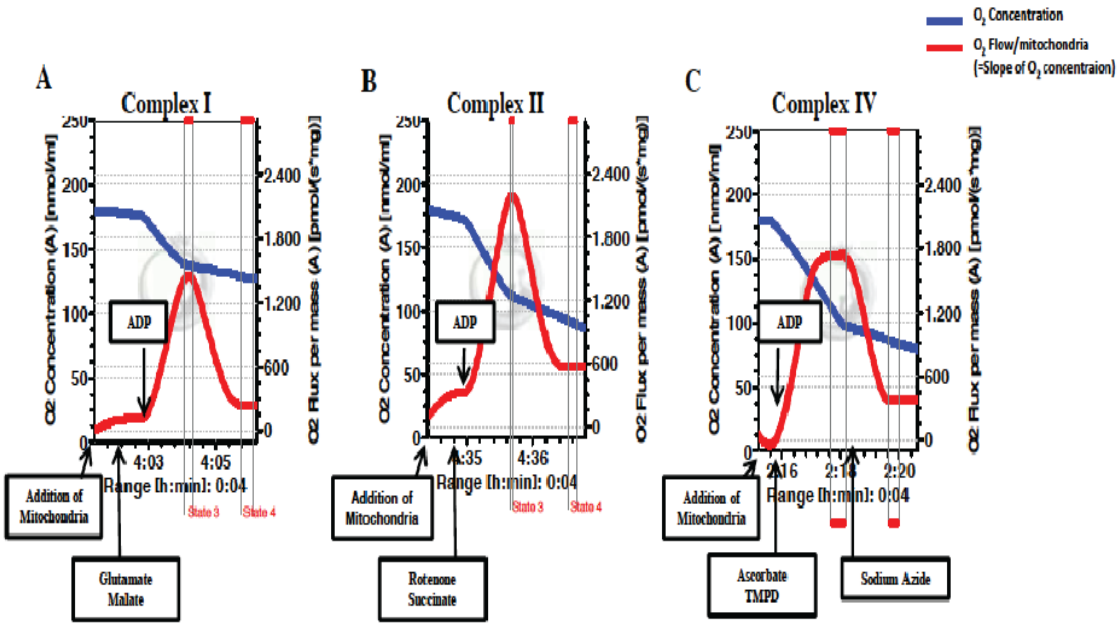

Figure 12. Representative traces of mitochondrial respiration form Oxygraph-2K. Isolated mitochondria were resuspended in respiration medium (Appendix 1.5) at a concentration of 0.4 mg/ml. Respiration rates were measured at 37ºC. (A) Complex I-dependent respiration was measured with glutamate (10 mM) plus malate (5 mM), followed by addition of ADP (0.5 mM). (B) Complex II-dependent respiration; first complex I was inhibited with rotenone (0.5 µM), and then succinate (10 mM) was added, followed by addition of ADP (0.5 mM). (C) Complex IV-dependent respiration was measured by adding ascorbate (4 mM) and TMPD (0.5 mM) followed by ADP (0.5 mM). Since TMPD exhibited a wide range of auto-oxidation in the buffer, respiration was finally inhibited with sodium azide (5 mM), and the difference between the oxygen consumption before and after the addition of sodium azide was interpreted as the real complex IV (State 3) respiration. The coupling of phosphorylation to oxidation was determined by calculating the respiratory control ratio (RCR) as the ratio between ADP-stimulated respiration (State 3) and respiration after ADP depletion (State 4).

2.8.4.2. ADP/O ratio

The 'P/O ratio' classically refers to the amount of ATP produced per oxygen atom reduced by the respiratory chain (ATP/O ratio). When using an oxygen electrode the amount of oxygen consumed on addition of a known amount of ADP (ADP/O ratio) can be used as a surrogate. It is a measure of the stoichiometry of the ATPase and that of O_2 reduction at complex IV and is therefore a constant for a given substrate (Hinkle 2005). It is therefore useful in assessing the 'health' of a mitochondrial isolate, but not in identifying (dys)function.

2.8.4.3. Respiratory acceptor control ratio (RCR)

The RCR is defined as the respiration rate in State 3 divided by that in State 4 (State 3/State 4). Healthy mitochondria display high respiratory control; they have the ability to markedly increase their rate of respiration in the presence of ADP to achieve high ATP output followed by a return to a resting low rate of respiration. A high RCR suggests that the mitochondria have high oxidative capacity with low proton leak. However, mitochondria from different tissues have different RCRs whilst the RCR for a given mitochondrion will vary depending on the substrate on which it respires. Importantly, any change in mitochondrial oxidative phosphorylating ability will be reflected in an altered RCR, making it a sensitive marker of mitochondrial (dys)function. If absolute rates of respiration and RCR are unaffected by an experimental condition it is unlikely that any bioenergetic dysfunction exists.

2.8.5. Isolation of functionally intact mitochondria

To prove that the isolation of crude mitochondria provided functionally intact organelles, respiration states of isolated rat liver mitochondria were measured (Fig. 13).

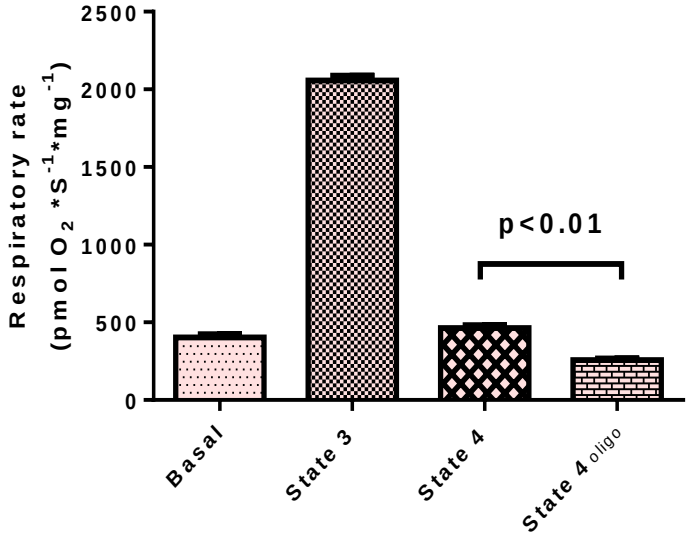

Figure 13. Respiratory rates of isolated rat liver mitochondria. Mitochondria were suspended in respiration medium (Appendix 1.5) with pyruvate and malate. State 3 respiration was measured in the presence of a saturating concentration of ADP. State 4 respiration was measured following the consumption of ADP. LEAK respiration was assessed after adding oligomycin (State 4_{oligo}). Data summarised from 3 runs in 3 different liver preparations (n=3). Data represent mean ± SEM. Differences were assessed with paired sample t tests.

	Basal	State 3	State 4	State 4_{oligo}	RCR_{oligo}	ADP/O
Mean	410.7	2164.5	495.2	289.0	7.7	3.1
SEM	29.4	101.4	20.2	4.7	0.4	0.4

Table 1 Respiratory rates in isolated rat liver mitochondria. Respiratory control ratio (RCR) was calculated using State 4 induced with oligomycin.

These data show that mitochondria isolated using the protocol described were functional and healthy. State 4 respiration rate was significantly higher than $State4_{oligo}$, suggesting extra-mitochondrial ADP regeneration likely due to presence of ATPases (Hütter et al. 2006). Taking $State4_{oligo}$ as the better indicator of true State 4, respiration

rates (State 3 and State 4) and RCR_{oligo} are within the expected ranges for mitochondria from 12-week old rat livers respiring on NADH linked substrates (Petrosillo et al. 2007; Long et al. 2006; Aprille & Asimakis 1980; Gold et al. 1968; Horton & Spencer 1981).

The ADP/O ratio is also within the range of published data though at the higher end of reported values for NADH-linked substrates in liver mitochondria (Hinkle 2005). This is likely to be due to a combination of factors. The lack of Mg^{2+} in the respiration medium may have contributed since this has been shown to slow the transition from State 3 to State 4 making estimation of the State 3 end point difficult (Hinkle & Yu 1979). This is owing to the fact that ATP is a competitive inhibitor of ADP transport whilst Mg-ATP is not (Pfaff & Klingenberg 1968). The use of a Teflon bar may have caused underestimation of O_2 consumption since it has been shown that Teflon adsorbs O_2 and releases it slowly during experiments with Clark electrodes (Hinkle et al. 1991). Finally there is disagreement in the literature regarding wither basal O_2 consumption should be deducted from calculations of ADP/O (Hinkle 2005).

Overall, however, these data describe functional and healthy mitochondria, with good reproducibility of respiration rate between assays, as evident in the small SEMs.

2.9. Statistical analyses

Data generated in the ligand binding studies and respirometry studies were subjected to statistical analyses.

Ligand binding data were analysed using the iterative non-linear curve fitting programmes Kinetic and LIGAND (KELL package, Elsevier Biosoft, Cambridge, UK). Parametric variables were reported as Mean (SD). Statistical significance (95% confidence interval) was reported for $p < 0.05$.

Statisistical analysis of respirometry data was performed using statistical software (Prism 5. Graphpad, CA. USA). Measures of central tendency for continuous variables were compared using Students' T test (parametric variables) following normality testing. For data comprising multiple groups, one-way analysis of variance (ANOVA) was used with Dunnett's test used for *post hoc* analysis. Parametric variables were reported as Mean (SEM). Statistical significance (95% confidence interval) was reported for $p < 0.05$.

3. RESULTS: ANALYSIS OF THE MITOCHONDRIAL PROTEOME

3.1. Analysis of the mitochondrial proteome by mass spectrometry

3.1.1. Western blotting crude mitochondrial fractions

In searching for components of the RAS within mitochondria, a proteomic survey of mitochondrial fractions isolated from rat liver was carried out using mass spectrometry. Crude mitochondria (CM) were isolated from a single rat liver and then separated into pure mitochondrial (PM) and mitochondrial associated membrane (MAM) fractions (as described in Methods, chapter 2.2). These whole fractions were run on a 10% Bis-Tris gel by SDS-PAGE.

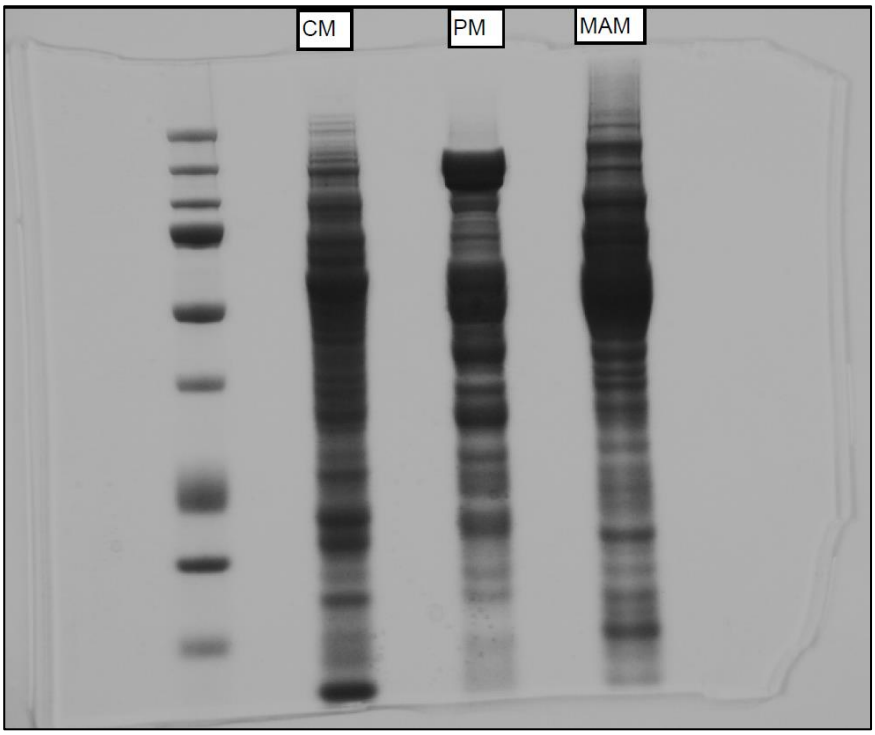

Figure 14. Coomassie Brilliant Blue stain of mitochondrial fractions run on SDS-PAGE. Each well was loaded with 40 µg protein and run on a 10% Bis-Tris gel. CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membranes.

The gel was cut into sections in preparation for mass spectrometry (Figure 15).

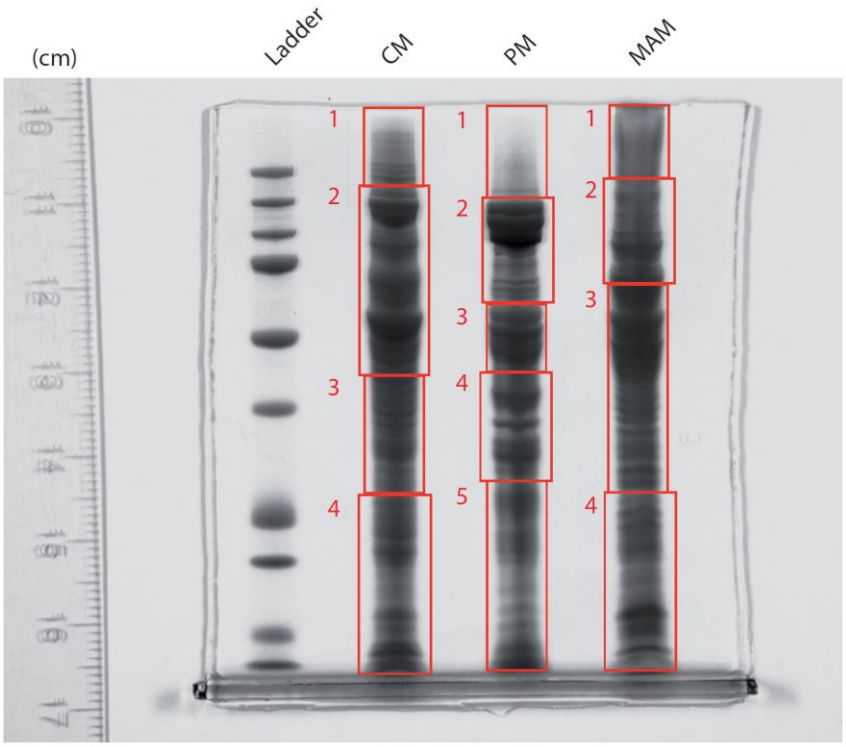

Figure 15. Coomassie Brilliant Blue stained acrylamide gel showing sectioning of lanes in preparation for mass spectrometry. Each lane was loaded with 40 µg protein and run on a 10% Bis-Tris gel. Lanes were cut into 4 or 5 sections and sent to the mass spectrometry facility for analysis. CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membranes.

Sections were cut from the 2D gel and sent to the Taplin mass spectrometry facility at Harvard Medical School (MA, USA). There, a dual source (MALDI and ESI) time of flight mass spectrometer (Orbitrap mass spectrometers, Thermo Scientific, MA, USA) was employed to identify peptides within the gel bands. Identified peptide sequences were cross matched with a protein database to provide protein matches. Any protein with three of more unique peptide matches was considered confidently identified in the sample. The Taplin Institute provided a list of all proteins that had any peptide sequence match, together with details of how many unique matches were found.

3.1.2. Results of mass spectrometry

Analysis of the 2-D gel sections returned hundreds of protein matches in each mitochondrial fraction (CM fraction contained 754 proteins; PM: 464 proteins; MAM: 713 proteins). To search for RAS components within this mitochondrial proteome, a list of all RAS related gene sets and their corresponding products was prepared using the AmiGO database (an online directory found at http://amigo.geneontology.org/amigo) which provides gene ontology details across species (see Appendix 2). The RAS related gene search encompassed biological processes and molecular functions. The proteins identified by mass spectrometry were cross referenced with the AmiGO gene list. To enable cross reference, proteins identified by mass spectrometry and proteins listed in the AmiGO gene set were given a common annotation as listed on the UniProt online directory (found at http://www.uniprot.org/). Intersections are listed in the following tables.

Crude mitochondria

UniProt term	Entry term	Protein name
P50123	AMPE_RAT	Aminopeptidase A
P54311	GBB1_RAT	Guanine nucleotide-binding protein subunit beta-1
P00787	CATB_RAT	Cathepsin B
P00786	CATH_RAT	Pro-cathepsin H

Table 2. RAS related protein intersections with crude mitochondrial proteome identified by mass spectrometry.

MAM

UniProt term	Entry term	Protein name
P50123	AMPE_RAT	Aminopeptidase A

Table 3. RAS related protein intersections with mitochondrial associated membrane (MAM) proteome identified by mass spectrometry.

Pure mitochondria

UniProt term	Entry term	Protein name
P50123	AMPE_RAT	Aminopeptidase A
Q68FT8	Q68FT8_RAT	Protein Serpinf2; Serine peptidase inhibitor
P00787	CATB_RAT	Cathepsin B
P00786	CATH_RAT	Pro-cathepsin H
P42676	NEUL_RAT	Neurolysin, mitochondrial

Table 4. RAS related protein intersections with pure mitochondrial proteome identified by mass spectrometry.

Only five RAS related protein matches were identified in the pure mitochondrial fraction, none of which are RAS specific. The least closely related is protein Serpinf2, a serine protease inhibitor and a negative regulator of endopeptidase activity that seems to be involved in RAS signalling pathways in smooth muscle, leading to contraction. However, it does not have any direct interaction with RAS effector peptides or receptors.

Cathepsin B is a cysteine endopeptidase with specificity for peptide bonds. It has been shown to catalyse the conversion of AngI to AngII in brain tissue (Azaryan et al. 1985) and is suggested to be involved in the intracellular processing of prorenin in cardiac tissue (Almeida et al. 2000). As such, it can perform the function of ACE whilst potentially also modulating renin availability. Cathepsin H is another member of the same class, and therefore capable of catalyzing similar reactions, though not documented to do so in the literature.

Aminopeptidase A is a recognized angiotensinase. It is a membrane bound metalloprotease responsible for hydrolyzing the NH_2 terminal aspartate of AngII to form AngIII (Erdös & Skidgel 1990; Zini et al. 1996). Initially identified in brain, it has since

been identified in other tissues including liver (Troyanovskaya et al. 1996; Gao et al. 2014; Lojda & Gossrau 1980). Whilst AngIII is metabolically active, its recognised effects are mediated through action at the AT_1R and AT_2R (Swindle et al. 2013; Yugandhar & Clark 2013; Clark et al. 2012; Kandalam et al. 2014), neither of which was identified in the pure mitochondrial or other fractions.

Neurolysin was also identified as a RAS related protein in the pure mitochondrial fraction. It is an oligopeptidase which was first identified in rat brain membrane as a novel neurotensin-degrading peptidase (Checler et al. 1986), and has since been identified in kidney, testis and liver amongst other tissues (Barelli et al. 1993; McKie et al. 1993; Serizawa et al. 1995). It has been shown to hydrolyze bradykinin and dynorphin A amongst other bioactive peptides (Swindle et al. 2013; Cavalcanti et al. 2014). However, it was noted to share sequence and binding characteristics with pig soluble angiotensin binding protein (endopeptise 24.15) (McKie et al. 1993; Kato et al. 1994) with the suggestion that it belonged to the same protein family, and therefore may be capable of binding AngII. Indeed, it has recently been identified as a non-AT_1R, non-AT_2R AngII binding site in brains of mice and humans (Karamyan et al. 2008; Wangler et al. 2012; Karamyan & Speth 2007) with functional cellular responses (Rashid et al. 2010; Bourassa et al. 2010). Moreover, It has been located on the inner membrane of rat liver mitochondria (Serizawa et al. 1995) raising the possibility of neurolysin acting as a novel mitochondrial AngII binding site.

However, such a role for neurolysin seems unlikely *in vivo.* First, the AngII binding property of neurolysin has only been shown *in vitro* under non-physiological conditions (requiring activation by p-chloromercuribenzoic acid (PCMB)) (Swindle et al. 2013). Further, data suggests that AngII may act to inhibit neurolysin rather than being its natural ligand (Barelli et al. 1994; Vincent et al. 1996). Finally, AngII binding to

neurolysin seems to be a brain-specific phenomenon; such binding is absent in rat liver homogenates (Karamyan & Speth 2007).

The mass spectrometric analysis of the mitochondrial proteome therefore failed to identify any canonical RAS components, nor a complete alternative system. Specifically, no recognised AngII receptors were identified.

However, this could represent a false negative finding. The coverage of the mitochondrial proteome by mass spectrometry of the mitochondrial fraction was incomplete. Using an existing database of recognised rat mitochondrial proteins (MitoMiner – see chapter 3.2), the proportion of the known proteome identified in the mitochondrial fractions can be calculated (Table 5).

	Rat MitoMiner	Mass Spec CM	Mass Spec PM	Mass Spec MAM
Number of proteins identified	1084	754	464	713
Number of proteins in intersection with Rat MitoMiner	-	330	301	244
Percentage coverage of MitoMiner proteins	-	30.4 %	27.8 %	22.5 %
Percentage of Mass spec proteins Mitochondrial	-	43.8 %	64.9 %	34.2 %

Table 5. Assessment of the coverage of the mitochondrial proteome by mass spectrometry. CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membranes.

Of the total number of known mitochondrial proteins in the rat proteome, only 27.8% were present in the pure mitochondrial fraction. There are multiple possible explanations for this apparently low proteome coverage. Loss of mitochondrial proteins during the isolation of pure mitochondria could contribute, though given that the crude mitochondrial fraction only had 30.4% coverage it would appear that protein loss occurs before pure mitochondrial separation.

Alternatively, the insensitivity of mass spectrometry may have contributed to the failure to detect proteins that had been isolated. A number of problems can occur before and during mass spectrometric analysis, including poor protein solubility, selective adsorption, ion suppression, selective ionization and cleavage into very short peptides (Aebersold & Goodlett 2001).

Sample preparation may also have led to a loss in mass spectroscopy sensitivity. Separating peptides from such a heavily loaded gel matrix is likely to account for a large portion of lost data. Greater detection may have been achieved with greater separation of protein bands. This can be achieved by using different gel preparations, or by using alternative methods of separation, such as 3D separation. By employing such methods a greater proportion of the mitochondrial proteome may have been detected. That said, the RAS proteins of interest lay predominantly in the mid to lower molecular range, in the section of the gel with better band separation.

Finally, the MitoMiner database is gathered from a range of tissues and mitochondrial subpopulations; it is known that there is great plasticity in the mitochondrial proteome, both within a given population and between populations (such as exist in different organs and cell types) (Calvo & Mootha 2010) meaning that only a proportion of the total proteome will be expressed at any given time in rat liver mitochondria. Whatever the reason is, the low coverage allows for the possibility of RAS related proteins to be falsely reported as absent.

Interestingly, this analysis also describes the purity of mitochondrial fractions separated using differential centrifugation and Percoll density separation. Mitochondrial proteins were enriched in the pure mitochondrial fraction, but this fraction also contained 35% non-mitochondrial proteins. Inversely, the MAM was significantly contaminated with

mitochondrial proteins (34.2% of total proteins). Given the proximity of ER and mitochondrial outer membrane at the MAM, and the tethering that occurs between the two, this is perhaps not surprising, but has not been previously reported.

This also highlights the possibility that proteins identified as ‘mitochondrial’ on the basis of mass spectrometry of mitochondrial fractions may be false positive associations; 30% of proteins in the pure mitochondrial fraction were non-mitochondrial.

Whilst these data do not support the existence of a mitochondrial RAS, they cannot formally exclude this possibility, given the gaps in coverage of the mitochondrial proteome identified by SDS-PAGE separation and mass spectrometry.

3.2. Interrogation of existing *in silico* databases

Proteomics refers to the large scale characterisation of gene and cellular functions at the protein level. It combines various disciplines including mass spectrometry, light and electron microcopy with green fluorescent protein (GFP) and gene array experiments. Advances in the field of proteomics over recent years have allowed for large scale mapping of numerous organelles and even organisms (Aebersold & Mann 2003). Data describing the mitochondrial proteome in a range of tissues from various organisms (including humans) have been published. These data have been integrated into databases which can be interrogated for proteins of interest.

MitoCarta is an inventory of 1098 genes encoding proteins for which there is strong evidence of mitochondrial localization (Pagliarini et al. 2008). It encompasses both nuclear and mtDNA encoded proteins. It is generated from the mass spectrometric analysis of mitochondria isolated from 14 mouse tissues, with large scale GFP-tagging studies carried out to assess protein localisation. The human homologues to these genes are listed in the same database allowing for cross species comparison.

MitoMiner is a database which combines localization data from 51 large scale GFP-tagging and mass spectrometry studies, mitochondrial targeting sequence predictions and details of protein sequence and function provided by the UniProt database. The inventory includes data relating to 12 species, including rat and human.

A list of RAS related gene sets from all the selected species was prepared from the AmiGo database (Appendix 2). These genes were cross-referenced with the MitoCarta and MitoMiner datasets and intersections sought.

Rat RAS gene intersections

MitoMiner

UniProt term	Entry term	Protein name
P54311	GBB1_RAT	Guanine nucleotide-binding protein subunit beta-1
P42676	NEUL_RAT	Neurolysin, mitochondrial

Table 6. Rat RAS gene intersections.

Human RAS gene intersections

A. MitoMiner

UniProt term	Entry term	Protein name
P62873	GBB1_HUMAN	Guanine nucleotide-binding protein subunit beta-1
P29597	TYK2_HUMAN	Non-receptor tyrosine-protein kinase TYK2
Q9BYT8	NEUL_HUMAN	Neurolysin, mitochondrial

B. MitoCarta

UniProt term	Entry term	Protein name
P19099	C11B2_HUMAN	Cytochrome P450 11B2, mitochondrial (Steroid 18-hydroxylase) (Aldosterone synthase)
Q9BYT8	NEUL_HUMAN	Neurolysin, mitochondria

Table 7. Human RAS gene intersections with (A) MitoMiner database and (B) MitoCarta database .

Mouse RAS gene intersections

A. MitoMiner

UniProt term	Entry term	Protein name
P62874	GBB1_MOUSE	Guanine nucleotide-binding protein subunit beta-1
Q91YP2	NEUL_MOUSE	Neurolysin, mitochondrial

B. MitoCarta

UniProt term	Entry term	Protein name
P15539	C11B2_MOUSE	Cytochrome P450 11B2, mitochondrial (Steroid 18-hydroxylase) (Aldosterone synthase)
G3UWE4	G3UWE4_MOUSE	Cytochrome P450 11B2, mitochondrial, isoform CRA b
Q91YP2	NEUL_MOUSE	Neurolysin, mitochondrial
P54320	ELN_MOUSE	Elastin (Tropoelastin)

Table 8. Mouse RAS gene intersections with (A) MitoMiner database and (B) MitoCarta database.

Bovine RAS gene intersection

MitoMiner

UniProt term	Entry term	Protein name
P62871	GBB1_BOVIN	Guanine nucleotide-binding protein subunit beta-1
P54392	NEUL_BOVIN	Neurolysin, mitochondrial

Table 9. Bovine RAS gene intersections with MitoMiner database.

With regard to MitoMiner RAS gene intersections, two relatively non-specific relations of the RAS were identified. First, the beta subunit of guanine nucleotide-binding protein (a G protein) was identified in all species. The beta chain is required for GTPase activity and for G protein-effector interactions and is therefore requisite for all G protein coupled receptors. As such, this protein is not specific for AngII receptors. Second, the non-receptor tyrosine protein kinase was identified as a human RAS gene/MitoMiner intersection. It is involved in intracellular signal transduction of multiple ubiquitous pathways including, but not exclusive to, some of those triggered by AT_1R and AT_2R.

Cross reference with MitoCarta identified cytochrome P450 11B2 as a RAS related mitochondrial protein in both humans and mice. This enzyme is involved in aldosterone synthesis (its alternative name is aldosterone synthase), converting 11-deoxycorticosterone into corticosterone, 18-hydroxycorticosterone and aldosterone, and as such is a target of the RAS, but is not an integral RAS protein.

Both MitoMiner and MitoCarta identified neurolysin as a mitochondrial RAS related protein. However, as discussed on page 81 there is no evidence for this protein acting as an AngII binding site or enzyme *in vivo* in rat liver.

As such, no integral members of the RAS were identified in the existing databases of mitochondrial associated gene products. These data support the results of mass spectrometry of SDS-PAGE separated mitochondrial fractions and make the existence of a mitochondrial RAS less likely. However, the existing databases on which both these analyses are based cannot exclude the presence of novel RAS component (for instance, a novel AngII binding site).

4. RESULTS:

IDENTIFICATION OF MITOCHONDRIAL ANGII RECEPTORS

4.1. Introduction

The concept of a functional mitochondrial RAS encompasses systems ranging from a complete intramitochondrial RAS, to an intramitochondrial action of imported RAS effector peptides, to an extramitochondrial system with direct action on the mitochondrion. In all these systems, RAS effector peptides must bind at the mitochondrion to effect their action. Identifying a binding site would confirm the potential for a functional mitochondrial RAS, whilst the location of this binding site on the inner or outer mitochondrial membrane would allow inference regarding the nature of the RAS. Immunochemistry is a well established technique for identifying and localizing proteins of interest in biological samples. However, this requires the selection of particular antigens of interest. With regards the RAS, this means selecting which of the known AngII receptors would be sought in mitochondria.

Of the established RAS receptors, the AT_1R has greatest binding affinity for AngII followed by the AT_2R, whilst AngII is a weak agonist at the AT_4R (which is not known to be expressed in rat liver (de Gasparo et al. 2000)). Both AT_1R and AT_2R are evolutionarily conserved (Auzan & Clauser 2009) and their expression has been reported on hepatic plasma and nuclear membranes (Tang et al. 1992; Booz et al. 1992; Eggena et al. 1993; Eggena et al. 1996; Abadir et al. 2011) making these the most likely mitochondrial AngII receptors.

Rodents express two AT_1R subtypes; AT_{1A} and AT_{1B} (Sandberg et al. 1994; Kakar et al. 1992; Iwai & Inagami 1992). They share 92-95% sequence homology and are pharmacologically indistinct (Speth et al. 1995; Guo & Inagami 1994). AT_{1A} transcripts are predominantly expressed in all tissues except the adrenal and pituitary gland where the AT_{1B} is the major subtype (de Gasparo et al. 2000). In the rat liver, reverse transcriptase-polymerase chain reaction (RT-PCR) evidence suggests that the relative

abundance of AT_{1A} is 100% (Llorens-Cortes et al. 1994). Functionally the two subtypes are indistinguishable (Gasc et al. 1994). Therefore AT_{1A} and AT_{1B} will be jointly referred to as AT_1R.

Review of the literature does provide immunochemical evidence of a mitochondrial binding site for AngII. Immunolabelled AngII localises to the mitochondria of rat adrenal, liver and brain tissue (Erdmann et al. 1996) where, in the rat sensory vagal cortex at least, immunolabelled AT_1R have been visualized (Huang et al. 2003). Meanwhile, immunofluorescent-tagged AT_2R have been reported to co-localise with the inner mitochondrial membranes in rat heart and liver (Abadir et al. 2011).

Therefore the presence of AT_1R and AT_2R was sought in subcellular fractions from rat lvier, including isolated mitochondrial inner and outer membranes.

4.2. Immunoblotting for mitochondrial AngII receptors

When incubating a membrane with primary antibody, a number of factors may influence its specific sensitivity, i.e. how effective that antibody is in recognising the protein of interest. Antibody concentration, incubation environment (in particular, temperature) and incubation time are all influential variables. The type of primary antibody employed is also significant.

There are broadly two types of primary antibody; monoclonal and polyclonal. Monoclonal antibodies (MAbs) are produced from a single B lymphocyte cell line *in vitro* (usually derived by fusing mammalian spleen cells with myeloma cells). Antibodies produced from these identical cloned cells have monovalent affinity; they all recognise the same epitope of the antigen. Polyclonal antibodies (PAbs) are typically produced by inoculating a suitable mammal with antigen which causes a polyclonal B lymphocyte proliferation producing a range of IgG peptides specific for various epitopes of the antigen. Many other nonspecific antibodies are present in the serum of these animals, so that on harvesting, affinity purification is used to isolate the antibodies of interest.

There are advantages and disadvantages to using MAbs and PAbs. PAbs often have better sensitivity due to the fact they are generated from a large number of B lymphocyte clones each producing antibodies to a (different) specific epitope. However, affinity purification often fails to clear all background nonspecific antibodies which can lead to non-specific background signals on Western blotting. MAbs are highly specific with high purity but since they only recognise a single epitope, their sensitivity is decreased if there are changes in the structure of the protein (for instance, truncation, genetic polymorphism, glycosylation or denaturation) (Lipman et al. 2005).

4.2.1. Immunoblotting for AT_1R in rat liver subcellular fractions

4.2.1.1. Antibody screen

Three commercially available antibodies to AT_1R were tested against subcellular fractions. SDS-PAGE Western blots were performed and proteins transferred onto PVDF membranes. Each antibody was applied for 16 hours at 4 ˚C and chemilluminescence was measured as described (Methods 2.3.2). The results of these blots are summarised in Table 10.

Company	Antibody	Ig Type	Dilution	Result
Abcam	**Ab 9391**	**MAb**	**1:200**	**No bands detected**
Sigma Aldrich	**HPA003596**	**PAb**	**1:1000**	**Multiple weak bands at various weights**
Santa Cruz	**Sc-1173**	**PAb**	**1:1000**	**Weak bands including at expected weight**
			1:500	**Stronger bands at expected weight**
			1:200	**Well defined bands at expected weight**

Table 10. Results of immunoblotting with three antibodies to AT_1R. Ig; immunoglobulin, MAb; monoclonal antibody, PAb; polyclonal antibody.

AT_1R is expressed on rat hepatocyte cell plasma and nuclear membranes, corresponding to the homogenate and nuclear fractions. The failure of the MAb (Ab 9319) to detect any bands in these fractions suggests either an altered conformation of the AT_1R leading to a change in the specific epitope that the MAb was raised against, or a failure in the incubation conditions. The 1:200 dilution used is a higher concentration than generally needed to detect proteins with a MAb, though if AT_1R is expressed in low concentration this may lead to failure of detection. A change in the AT_1R confirmation cannot be ruled out.

The multiple bands resolved with HPA003596 at various molecular weights suggest cross reactivity with non-target proteins, likely a consequence of large amounts of non-specific antibody in the antiserum ineffectively cleared by affinity purification.

Sc-1173 gave the most promising results and was used for all subsequent immunoblotting at a concentration of 1:200, applied for 16hours in 5% BSA at 4 ˚C.

4.2.1.2. SDS-PAGE Western blots for AT_1R in rat liver subcellular fractions

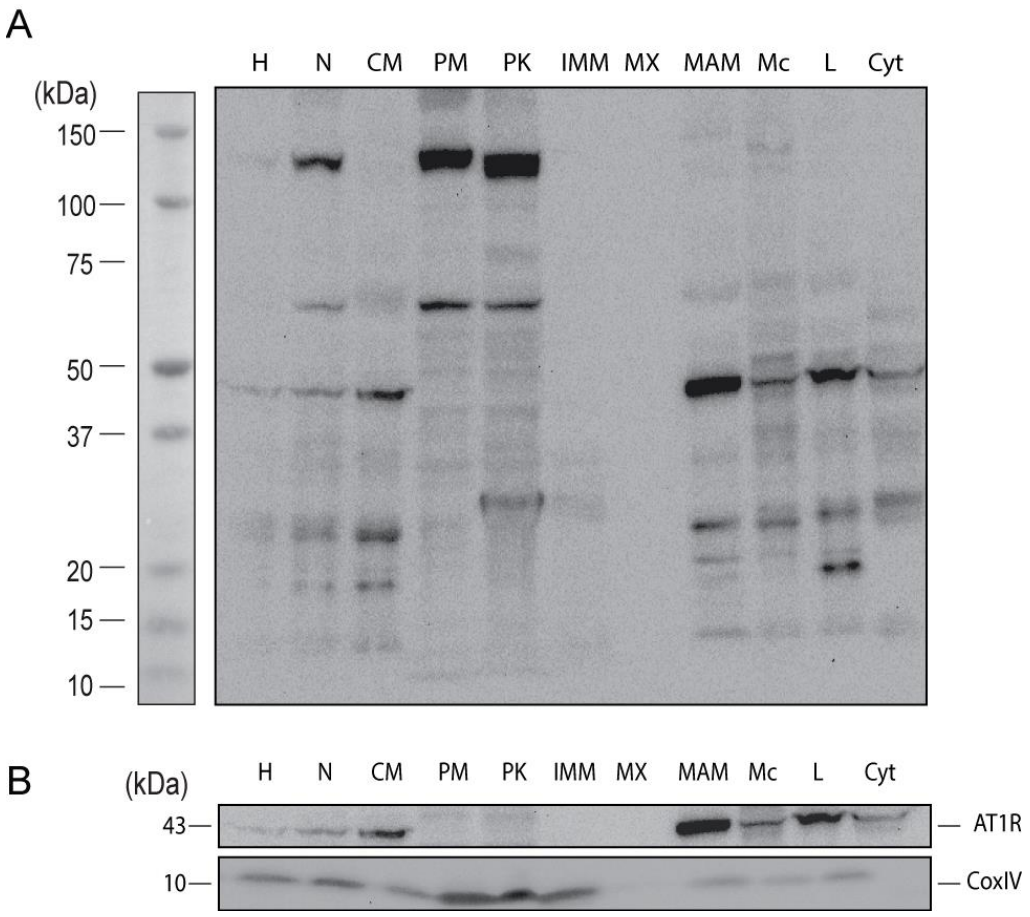

Figure 16. Western blot detection of AR_1R in subcellular fractions. (A) Sc-1173 produced a band at approximately 43 kDa in the CM and MAM fractions but not the PM. (B) CoxIV was employed as a mitochondrial marker. H; homogenate, N; nuclear, CM; crude mitochondria, PM; pure mitochondria, PK; proteinase K digest, IMM: inner mitochondrial membranes, Mx; mitochondrial matrix, MAM; mitochondrial associated membrane, Mc; microsomes, L; lysosomes, Cyt; cytosol.

Employing Sc-1173 as the primary antibody revealed a band at approximately the expected molecular weight of AT_1R (43 kDa). This band was present in increasing density in the homogenate, nuclear and crude mitochondrial fractions, in keeping with a mitochondrial protein. However, the 43 kDa band was not present in any of the pure mitochondrial fractions (pure mitochondria, proteinase K digest, inner mitochondrial membranes or mitochondrial matrix) but an enriched band in the MAM fraction was observed. There was also a band visible in the lysosomal fraction and to a lesser extent

the microsomal and cytosolic fractions. There was minor mitochondrial contamination, evidenced by the very faint CoxIV bands in non-mitochondrial subfractions.

Further blots were performed on subcellular fractions isolated from three separate rat livers (n = 3) excluding mitochondrial subfractions, allowing for greater enrichment of the PM and MAM fraction.

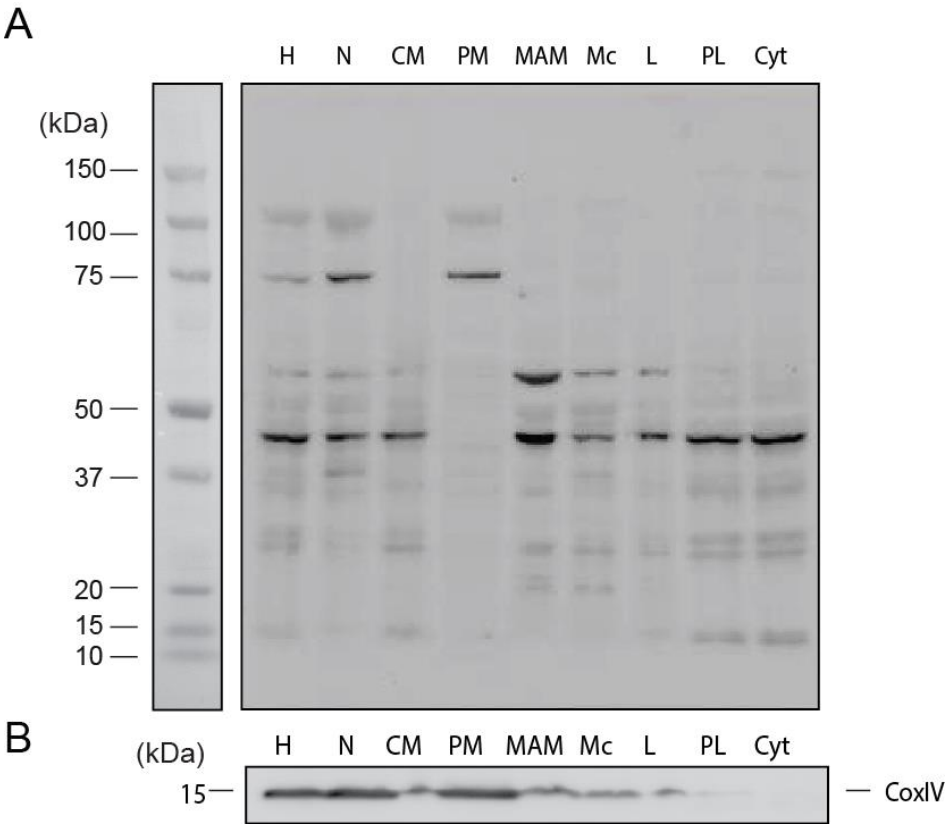

Figure 17. Representative blot showing immunostaining for AT_1R with Sc-1173. Immunoreactive bands at the expected weight of AT_1R (43 kDa) were present in all fractions other than the pure mitochondria. CoxIV was used as a mitochondrial marker. H; homogenate, N; nuclear, CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membrane, Mc; microsomes, L; lysosomes, PL; post-lysosomal supernatant, Cyt; cytosol.

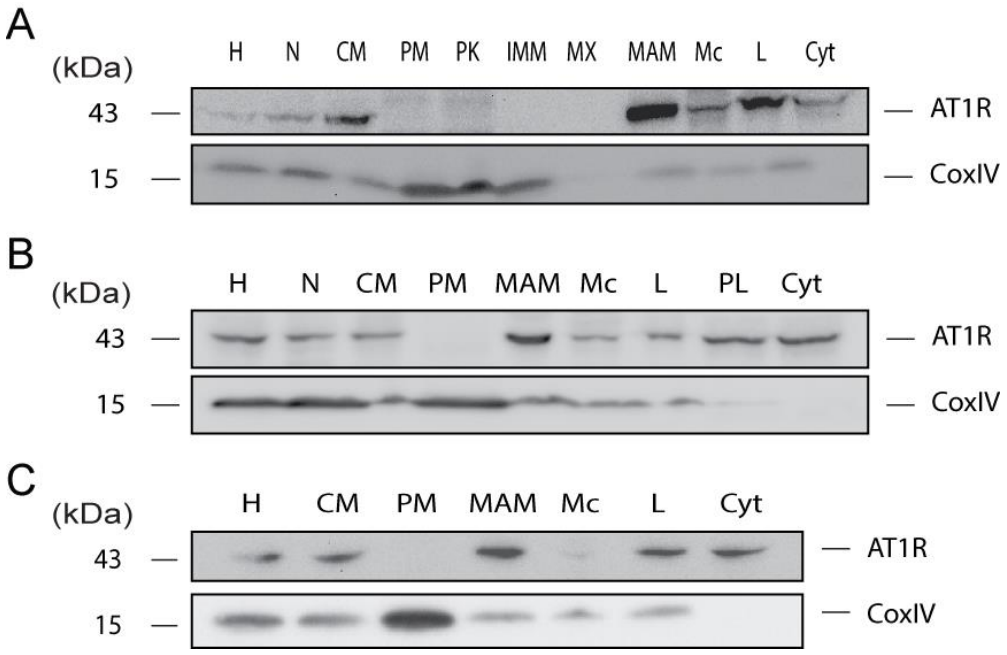

Figure 18. Composite figure of AT_1R staining patterns in 3 liver preparations. Subcellular fractions from 3 livers (A, B and C, n=3) were run on SDS-PAGE and immunostained for AT_1R with Sc-1173. Immunostaining with CoxIV as a mitochodnrial marker was carried out on each membrane. H; homogenate, N; nuclear, CM; crude mitochondria, PM; pure mitochondria, PK; proteinase K digest, IMM; inner mitochondrial membranes, Mx; mitochondrial matrix, MAM; mitochondria associated membrane, Mc; microsomes, L; lysosomes, PL; post-lysosomal supernatant, Cyt; cytosol.

In all three blots the same pattern of AT_1R staining was seen, with bands at 43 kDa in CM and MAM fractions but not in the PM fraction. CoxIV staining confirmed adequate mitochondrial protein in the PM lane. This suggests AT_1R presence in the MAM but not in pure mitochondria.

4.2.1.3. Validation of Sc-1173 specificity for AT_1R

In order to draw conclusions regarding AT_1R subcellular location based on Western blots with Sc-1173, the specificity of the antibody was tested using a selective AT_1R small-interfering RNA (siRNA). Successful silencing of the AT_1R gene results in a decreased expression of AT_1R protein which should be reflected on Western blots as a decreased intensity of the 43 kDa band seen with Sc-1173.

Cell cultures of HepG2 cells (of human hepatocellular origin) in 6 well plates were incubated with AT_1R siRNA (#29750, Santa Cruz, Texas, USA) non-targeting siRNA (#D0012100205, Dharmacon, GE Healthcare, Buckinghamshire, UK) , or no siRNA (untransfected) as described in Methods section 2.6.2. Selective p53 siRNA (#29435, Santa Cruz, Texas, USA) was used as a control for successful transfection. Cells were harvested at 48 hours post transfection and whole cell lysates prepared for SDS-PAGE Western blotting using Sc-1173 to immunoblot AT_1R and Sc-DO-1 for p53. β-actin (Sc-47778) was employed as a loading control. All antibodies were supplied by Santa Cruz (Texas, USA).

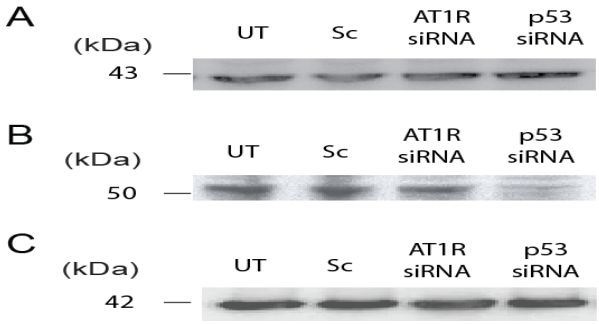

Figure 19. Western blots of AT_1R and p53 siRNA silencing in HepG2 cells. (A) AT_1R gene silencing; immunoblotting with Sc-1173 revealed bands at 43 kDa in the untransfected (UT) and scrambled siRNA (Sc) samples. In cells exposed to selective AT_1R siRNA no decrease in the intensity of the 43 KDa band was seen. (B) p53 gene silencing; immunoblotting with Sc-DO-1 revealed successful p53 gene silencing reflected in the decreased intensity of the 50 kDa band in cells exposed to the specific p53 siRNA. (C) Loading control using antibody against β-actin revealed equal loading in all wells.

There are a number of possible explanations for these data. First, transfection of AT_1R siRNA may not have been successful. This possibility is made less likely by the successful gene silencing of p53 using the same protocol suggesting the transfection protocol was effective. Secondly, the time allowed for silencing of the AT_1R gene by successfully transfected siRNA (48 hours) may have been insufficient. Refuting this argument are data showing that in Human Embryonic Kidney 293 (HEK293) and immortalized Proximal Tubule Cells (PTCs) AT_1R protein is down-regulated in response to AT_1R siRNA within 24 hours and maximally at 48hrs (Li & Zhuo 2007) whilst in HepG2 cells transfected with AT_1R siRNA (Santa cruz SASI_Hs02_00206672) and harvested at 48 hours, an 80% reduction in AT_1R mRNA was demonstrated (Nabeshima et al. 2009). An alternative explanation in that AT_1R protein may have been successfully down regulated but this was not apparent due to non-specificity of Sc-1173.

4.2.2. Immunoblotting for AT_2R in rat liver subcellular fractions

4.2.2.1. Antibody screen

Two candidate commercial antibodies raised against AT_2R were tested in an antibody optimisation process. Subcellular fractions were run on SDS-PAGE and transferred to PVDF membranes. Each antibody was incubated with the membrane in 5% BSA for 16 hours at 4 ˚C. The predicted weight of AT_2R is 41 kDa, though the observed *in vitro* weight is closer to 50 kDa.

Company	Antibody	Ig Type	Dilution	Result
Abcam	Ab 19134	PAb	1:200	Weak bands detected with none at 41- 50 kDa
Santa Cruz	Sc-9040	PAb	1:200	Multiple intense bands detected including at 41 - 50 kDa

Table 11. Results of immunoblotting with two antibodies to AT_2R (expected MW 41 - 50 kDa). Ig; immunoglobulin, MAb; monoclonal antibody, PAb; polyclonal antibody.

Ab 19134 displayed non specificity and poor sensitivity. As such, Sc-9040 was chosen for all further blots.

4.2.2.2. SDS-PAGE Western blots for AT_2R in rat liver subcellular fractions

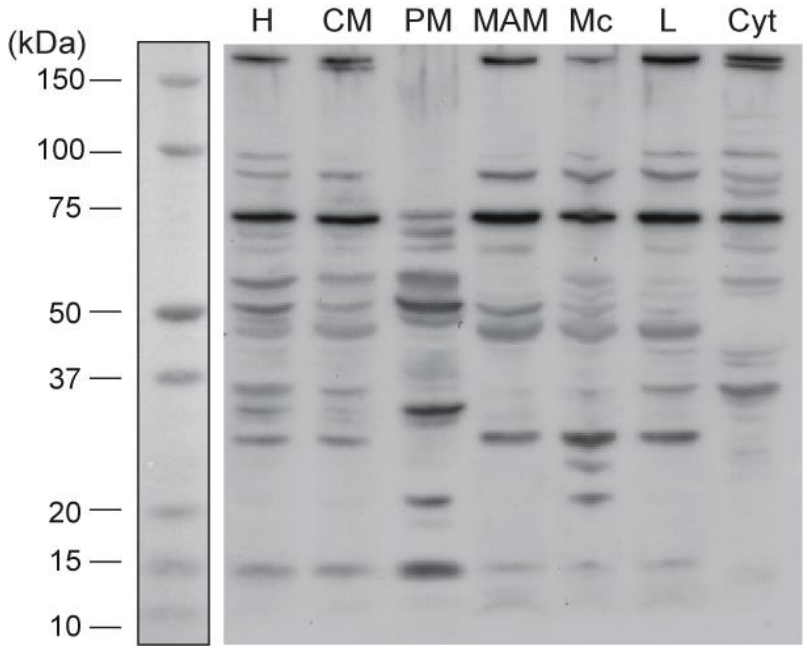

Figure 20. Representative blot showing immunostaining for AT_2R with Sc-9040. Multiple bands were detected in all fractions including bands at the approximate weight of AT_2R (41 KDa). H; homogenate, CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membranes, Mc; microsomes, L; lysosomes, Cyt; cytosol.

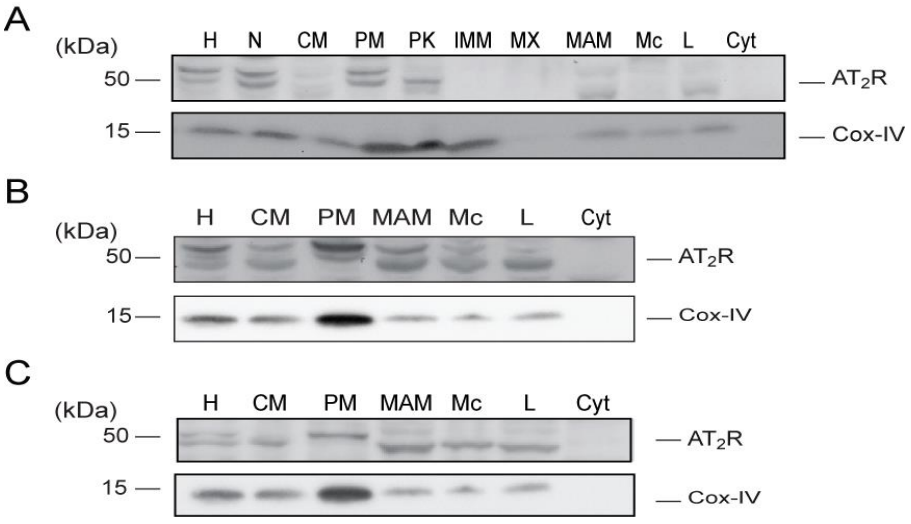

Figure 21. AT_2R panels from 3 separate liver isolations. Images have been cropped to include all immunoreactive bands around the expected weight of AT_2R (41 kDa). H; homogenate, N; nuclear, CM; crude mitochondria, PM; pure mitochondria, PK; proteinase K digest, IMM; inner mitochondrial membranes, Mx; mitochondrial matrix, MAM; mitochondria associated membranes, Mc; microsomes, L; lysosomes, Cyt; cytosol. CoxIV was used as a mitochondrial marker.

The staining pattern with Sc-9040 suggests low specificity of the polyclonal antibody, or the contaminating presence of antibodies directed against non-target proteins. Interpreting these blots further is therefore unsafe.

4.3. Identification of immunoreactive proteins by mass spectrometry

4.3.1. Immunoprecipitation of mitochondrial AngII receptors

Definitive identification of proteins in immunoreactive bands visualized with both Sc-1173 (AT_1R) and Sc-9040 (AT_2R) was sought by mass spectrometry of the SDS-PAGE gel bands.

The detection limit of a mass spectrometer for a particular protein is governed by the molar mass of that protein and is generally in the femtomole range. For proteins less than 75 kDa this corresponds to 2-5 nanograms. It is very common for gel bands to contain many different proteins, so that the protein of interest may be present in only small amounts. Increasing the amount of protein loaded in each well would help in this situation, but this is limited by the volume capacity of each well. Immunoprecipitation of proteins affords a way in which to purify the protein of interest, allowing an increased amount to be run on gels and thus increasing the sensitivity of detection with mass spectrometry.

Subcellular fractions (400 µg) isolated from the livers of 12-week old male Sprague-Dawley rats were used as input in the immunoprecipitation protocol (see Methods chapter 2.5). The supernatant from each input was incubated with Sc-1173 or Sc-9040 for 16hours at 4 ˚C. The elute produced was run on SDS-PAGE.

4.3.1.1. Immunoprecipitation of AT_1R with Sc-1173

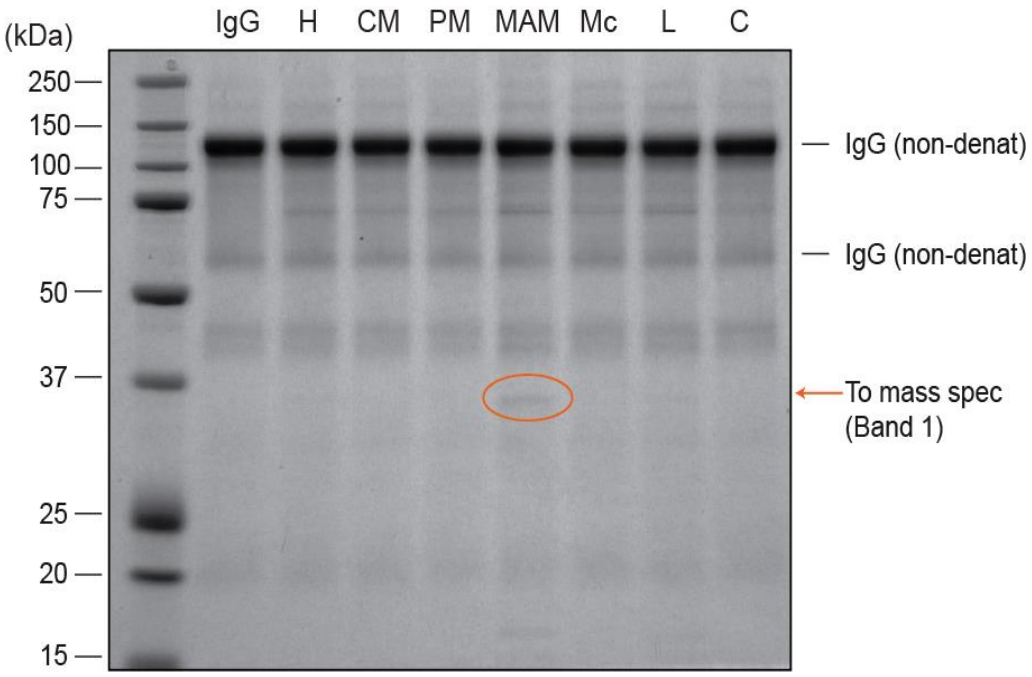

Figure 22. Coomassie Brilliant Blue stain of 10% Bis-Tris gel following immunoprecipitation of AT_1R from subcellular fractions with Sc-1173. Each lane was loaded with 40 µg of immunoprecipitant. A single band is visible in the MAM fraction near the expected weight of AT_1R (43 KDa) which does not appear in the other fractions. H; homogenate, CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria associated membranes, Mc; microsomes, L; lysosomes, and C; cytosol.

The heavily stained band at c.120 kDa is due to non-denatured IgG present in the sample, whilst the heavy chain of the Ig appears at 75 kDa and the light chain at 20 kDa. A well-defined band is seen in the MAM fraction just below 37 kDa. To increase the quantity of protein in this band, further immunoprecipitation was conducted in the MAM fraction alone, using a greater amount of MAM as the input and loading 50 µg immunoprecipitant in the well of the gel.

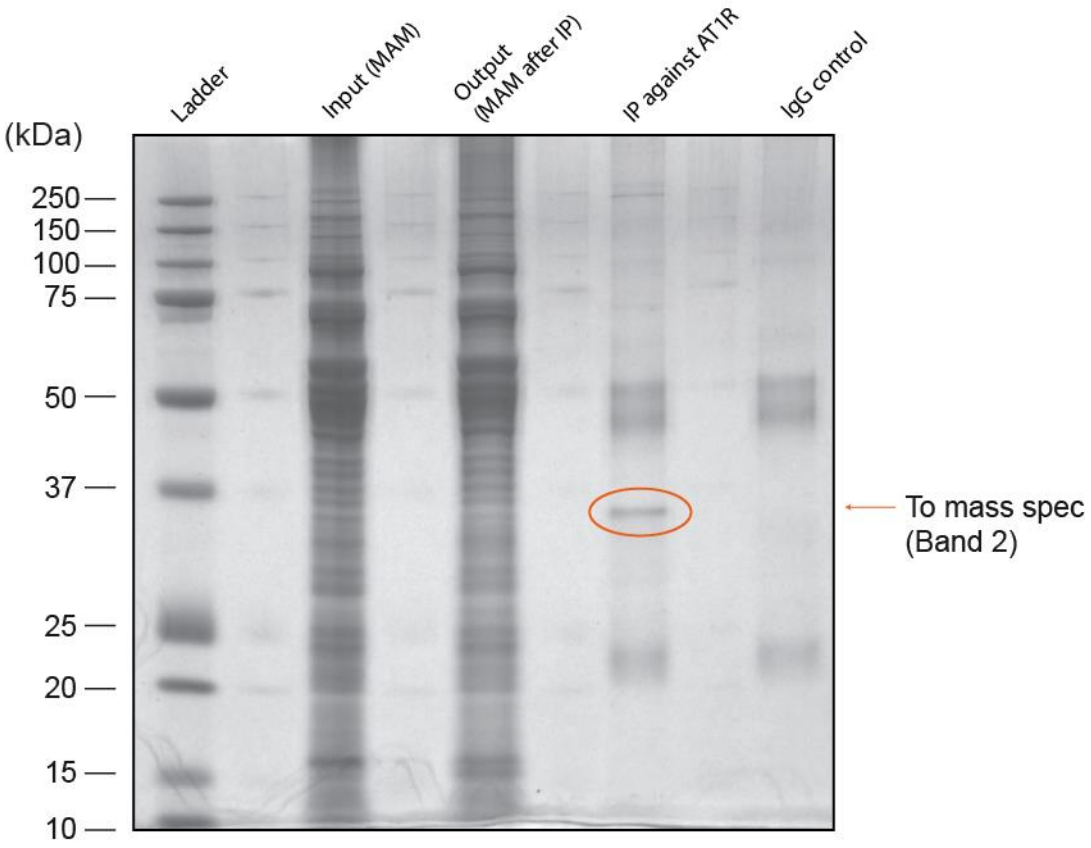

Figure 23. Coomassie Brilliant Blue staining of 10% Bis-Tris gel following immunoprecipitation of AT_1R from MAM with Sc-1173. MAM; mitochondria associated membrane, IP; immunoprecipitation.

On immunoprecipitation of AT_1R from the MAM, a single band was seen at approximately 35 kDa. This band was present in the input and absent in the output, in keeping with effective immunoprecipitation. The weight of the band is lower than the expected weight of AT_1R (43 kDa). However, truncated forms of AT_1R have been described (Cook & Re 2009; Cook et al. 2007; Conchon et al. 1999) and a cleavage site has been identified that cleaves the complete receptor into 6 kDa and 37 kDa fragments (Cook et al. 2011) suggesting this band could be a truncated form of the receptor.

Both bands were cut from the 2D gel and sent for analysis at the Taplin mass spectrometry facility at Harvard Medical School (MA, USA).

4.3.1.2. Immunoprecipitation of AT_2R with Sc-9040

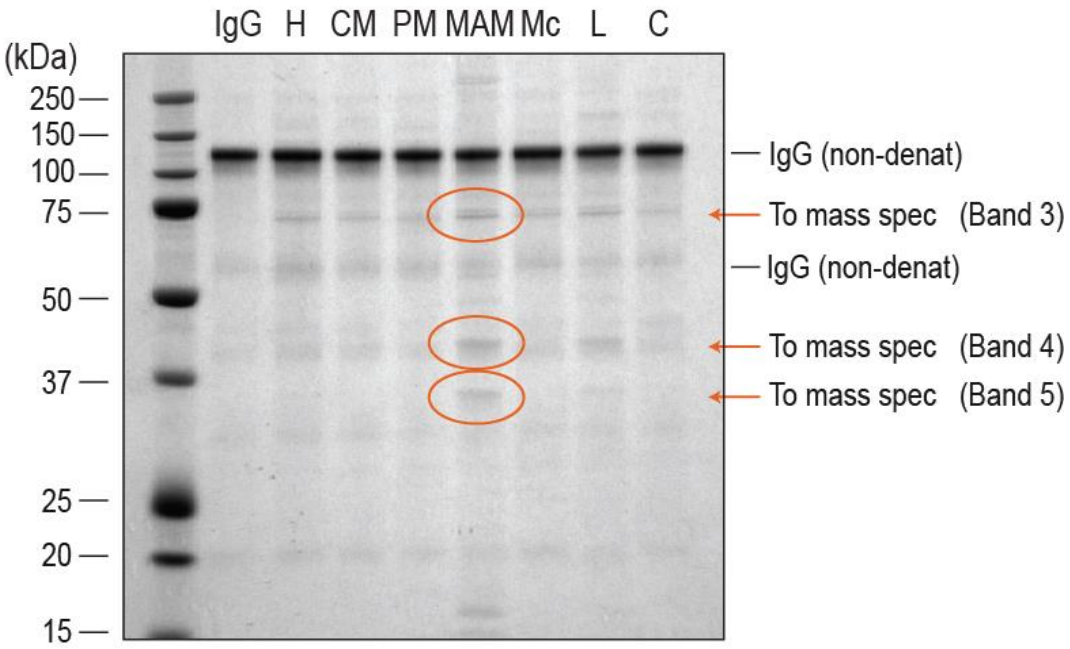

Figure 24. Coomassie Brilliant Blue staining of 10% Bis-Tris gel following immunoprecipitation of AT_2R from subcellular fractions with Sc-9040. Each lane was loaded with 40 µg of immunoprecipitant. H; homogenate, CM; crude mitochondria, PM; pure mitochondria, MAM; mitochondria assocaited membranes, Mc; microsomes, L; lysosomes, and C; cytosol.

Following immunoprecipitation of AT_2R using Sc-9040, excluding bands accounted for by IgG heavy and light chains, three distinct bands were seen. Bands at 75 kDa (band 3) and 40 kDa (band 4) were present in all fractions, whilst a band just below 35 kDa (band 5) was visible in MAM only.

All three bands from the MAM fraction were cut from the gel and analysed by mass spectrometry at the Taplin mass spectrometry facility at Harvard Medical School (MA, USA).

4.3.2. Mass spectrometry of AT_1R gel bands

Gel fragments were analysed by mass spectrometry (Taplin biological mass spectrometry facility, Harvard medical school, MA, USA). Any protein with three or more unique peptide matches was considered 'confidently' identified in the sample. Proteins with less than three unique peptide matches are not considered to be confidently identified without further confirmation.

Protein name	Unique peptide matches	% coverage	Location	Function
Uricase	20	48.5%	Peroxisome/mitochondrion	Catalyzes the oxidation of uric acid to 5-hydroxyisourate, which is further processed to form (S)-allantoin.
60S acidic ribosomal protein P0	2	6.9%	Cytoplasm/nucleus	Ribosomal protein
Actin, aortic smooth muscle	2	6.9%	Cytoplasm/nucleus	Actins are highly conserved proteins, are involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells.
Tubulin beta-2A chain	1	2.2%	Cytoplasm	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain

Table 12. Mass spectroscopy of gel band 1. Proteins with ≥ 3 unique peptide matches are considered to be identified with confidence. No AT_1R related peptides were identified.

Protein name	Unique peptide matches	% coverage	Location	Function
Uricase	23	52.8%	Peroxisome/mitochondrion	Catalyzes the oxidation of uric acid to 5-hydroxyisourate
Peroxiredoxin-1	7	32.7%	Cytoplasm	Involved in redox regulation of the cell
Heterogeneous nuclear ribonucleoproteins A2/B1	5	15.6%	Cytoplasm/nucleus/spliceosome	Involved with pre-mRNA processing
60S acidic ribosomal protein P0	2	6.9%	Cytoplasm/nucleus	Ribosomal protein
Heterogeneous nuclear ribonucleoprotein A3	2	3.4%	Nucleus/spliceosome	Plays a role in cytoplasmic trafficking of RNA. Binds to the cis-acting response element, A2RE
Arginase-1	2	4.6%	Cytoplasm	Catalyses the reaction: L-arginine + H2O = L-ornithine + urea.
F-actin-capping protein subunit alpha-1	1	3.5%	Cytoplasm	F-actin-capping proteins bind in a Ca2+-independent manner to the fast growing ends of actin filaments

Protein name	Unique peptide matches	% coverage	Location	Function
Peroxiredoxin-4	1	4.0%	Cytoplasm/secreted	Probably involved in redox regulation of the cell.
Peroxiredoxin-2	1	5.6%	Cytoplasm	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system
L-lactate dehydrogenase A chain	1	2.7%	Cytoplasm	Catalyses the reaction: (S)-lactate + NAD^+ = pyruvate + NADH

Table 13. Mass spectroscopy of gel band 2. Proteins with ≥ 3 unique peptide matches are considered to be identified with confidence. No AT_1R related peptides were identified

4.3.3. Mass spectrometry of AT_2R gel bands

All three bands from the immunoprecipitated MAM fraction were analysed by mass spectrometry. No peptide matches were found in band 3 (at 75 kDa). Peptide matches in the other bands are details in tables 14 and 15.

Protein name	Unique peptide matches	% coverage	Location	Function
Uricase	8	23.8%	peroxisome/mitochondrion	Catalyzes the oxidation of uric acid to 5-hydroxyisourate, which is processed to form allantoin.

Table 14. Mass spectroscopy of gel band 4 (40 KDa band). Proteins with ≥ 3 unique peptide matches are considered to be identified with confidence. No AT_2R related peptides were identified.

Protein name	Unique peptide matches	% coverage	Location	Function
Actin, aortic smooth muscle	7	21.8%	cytoplasm/nucleus	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
Actin, cytoplasmic 1	4	12.0%	cytoplasm/cytoskeleton	Actins (see above)
Uricase	2	7.6%	peroxisome/mitochondrion	Catalyzes the oxidation of uric acid to 5-hydroxyisourate, which is processed to form allantoin.
40S ribosomal protein S9	2	8.8%	cytoplasm	Identified in a IGF2BP1-dependent mRNP granule complex containing untranslated mRNAs
Betaine--homocysteine S-methyltransferase 1	2	4.4%	cytoplasm	Involved in the regulation of homocysteine metabolism. Converts betaine and homocysteine to dimethylglycine and methionine, respectively.

Table 15. Mass spectrometry of gel band 5 (35 KDa band). Proteins with ≥ 3 unique peptide matches are considered to be identified with confidence. No AT_2R related peptides were identified.

4.4. Conclusions

In these experiments, the presence of a recognised AngII receptor was sought in rat liver mitochondria. Western blotting with specific antibodies was employed. Several antibodies against AT_1R were screened. The monoclonal antibody (ab 9391) did not reveal any protein at the expected weight of the receptor (43 kDa) whilst the polyclonal HPA003596 did show some chemiluminescent bands but they were at several molecular weights and the signal was weak. Optimisation of conditions using Sc-1173 produced a defined band at 43 kDa. This band was present in the crude mitochondrial fraction and the MAM but not in pure mitochondrial fractions. The specificity of the antibody could not be proven by siRNA silencing of the receptor and so mass spectrometry of the immunoprecipitated band was performed to confirm the presence of AT_1R peptides. No such peptides were found.

The pattern of immunoreactive bands visualized with the chosen AT_2R antibody (Sc-9040) suggested non-specificity, with multiple bands even in the region of the expected weight of the target protein. Immunoprecipitation produced bands at 75 kDa, 40 kDa and 35 kDa. The 75 kDa and 40 kDa bands were present in all subcellular fractions, whilst a 35 kDa band was only visualized in the MAM fraction. All three bands were subjected to mass spectrometry. No peptides were identified in the 75 kDa band whilst no AT_2R-specific peptides were identified at 40 kDa or 35 kDa.

The failure to identify AngII receptor proteins in the immunoreactive bands by mass spectrometry is either a ‘false negative’ result due to the abundance of the receptor falling below the sensitivity threshold of the spectrometer, or a ‘true negative’ due to non-specificity of the antibodies employed.

Immunoreactive bands were visualized in Tris-Bis gels using Coomassie brilliant blue staining which has a detection limit of approximately 50 ng. The sensitivity of

the mass spectrometer varies according to molar mass, but in general equates to 2-5 ng for proteins less than 75 kDa. As such, proteins clearly visualised with Coomassie brilliant blue should be detected in the mass spectrometer. Since the fractions run on the analysed gel contained only proteins selected by immunoprecipitation this suggests non-specificity of the antibodies.

The Sc-1173 immunoglobulin is an affinity purified rabbit polyclonal antibody. It is targeted against a non-specified peptide from the N-terminal extracellular domain of the human AT_1R, with specificity described for human, mouse and rabbit in applications including Western blot and immunoprecipitation. Many groups have published data using Sc-1173, though often without providing evidence of its specificity in their laboratories. Those that have provided evidence of specificity have relied upon pre-adsorption tests in which a synthetic peptide corresponding to the specific epitope recognised by the antibody is incubated with the antibody to block binding (as a negative control) (Harrison-Bernard et al. 1999; Zhuo, Carretero, et al. 2006; Zhuo, Li, et al. 2006). However, binding to the synthetic antigen does not equate to specificity for the receptor; Rateri *et al* showed strong binding of Sc-1173 to immunising peptide but were unable to demonstrate any specific interaction with the AT_1R (Rateri et al. 2011). This is particularly pertinent for antibodies that have already been affinity purified, since such an antibody has been produced by screening for its binding to the target epitope and will therefore always bind it and always pass an pre-adsorption test whether specific for the protein or not (Saper 2009).

An important study of the specificity of commercially available AT_1R antibodies, published after I had completed the work described, confirms my findings that many commercially-available antibodies may be highly non-specific in their binding characteristics. Benicky *et al* screened six antibodies including Sc-1193 and ab9391. Using AT_1R knockout animals (that do not express any AT_1R) they showed

all six antibodies recognise proteins of similar size to AT_1R, but produced identical immunostaining patterns in Western blots from wild-type and AT_1R knockout mice (Benicky et al. 2012). They concluded that none of the commercially available antibodies tested were specific for AT_1R in rodents. Similar conclusions were drawn in testing of newly developed non-commercial AT_1R antibodies (Rateri et al. 2011).

Similarly, there is now good evidence to suggest that commercially available AT_2R antibodies are also non-specific. Sc-9040 is a polyclonal antibody raised in rabbit against a protein corresponding to amino acids 221-363 of the AT_2R of human origin. It is quoted as having specificity in human, mouse and rat tissues in applications including Western blots and immunoprecipitation. In a study by Hafko *et al*, the specificity of Sc-9040 along with two other commercially available and commonly employed AT_2R antibodies was tested. In both mouse and rat tissue extracts multiple immunoreactive bands were observed and there was no correlation between the observed immunoreactivity and the presence or absence of the AT_2R protein, receptor binding or gene expression (Hafko et al. 2013).

On review of the available literature, there is no convincing evidence of mitochondrial AT_1R or AT_2R using antibodies with proven specificity. Of particular note, Abadir *et al* described mitochondrial AT_2R in liver using Sc-9040 for Western blots and an alternative antibody (sc 48451) for immunochemistry for which no evidence of specificity was provided.

With regards the presence of an AngII binding site in liver mitochondria, these data do not exclude this possibility. Specific antibodies to AT_1R and AT_2R may have enabled their isolation and identification with the methods employed here. Alternatively, a novel AngII receptor may be present. As concluded by Hafko and Benicky, in the absence of specific antibodies, the gold standard method for

identification of ligand binding sites is competitive radioligand binding assays (Benicky et al. 2012; Hafko et al. 2013).

5. RESULTS:

RADIOLIGAND

BINDING STUDIES

5.1. Introduction

Mass spectrometry of both the mitochondrial proteome and bands resulting from the targeted immunoprecipitation of AngII receptors failed to identify any mitochondrial AngII binding site. However, a receptor in low abundance may not have been detected by whole proteome mass spectrometry, whilst the non-specificity of AT_1R and AT_2R antibodies undermined the attempt to use immunochemistry to isolate them.

Radioligand binding assays provide an alternative way of investigating the presence and characteristics of any mitochondrial AngII binding site and are regarded as the gold standard technique in the study of membrane receptors (Jensen et al. 2009; Hafko et al. 2013; Benicky et al. 2012; Saper & Sawchenko 2003). Ligands with high specific activity allow receptors that are usually expressed in low densities to be characterized. Binding assays provide a quantification of the interaction between these ligands and their receptors, encompassing receptor binding affinity and population density. When specific receptor antagonists are available, radioligand binding assays can additionally identify receptor subtype.

Radioiodinated AngII ([^{125}I]-AngII) has been used for decades to investigate the binding properties of AngII in various tissues. There are some data reporting [^{125}I]-AngII binding in subcellular fractions. Sirett *et al* investigated subcellular [^{125}I]-AngII binding in rat brain homogenates and reported 18.2% of total specific binding in the mitochondrial fraction (Nancy E. Sirett et al. 1977) whilst van Kats *et a*l reported [^{125}I]-AngII binding in the mitochondrial fractions of pig kidney and adrenal glands (van Kats et al. 2001). However, these studies were performed with crude mitochondrial fractions and so do not clarify whether AngII binds to mitochondria themselves or to associated membranes (MAM).

Radioligand binding studies were carried out with subcellular fractions, first to confirm binding of AngII in crude mitochondria, then to clarify whether this binding occurred at the mitochondrion or MAM.

These studies were carried out in collaboration with Dr Anthony Davenport of the Clinical Pharmacology Unit, University of Cambridge with the direction of the work determined through regular discussions between us. I prepared all membrane samples at University College London and performed protein quantification. Due to the highly skilled nature of this technique, Dr Davenport and his colleague Dr Rhoda Kuc then performed the experiments.

5.2. Optimisation of ligand binding assays

For saturation assays, the concentration of [^{125}I]-AngII had to be chosen empirically since no data exist regarding the affinity of binding to liver mitochondrial fractions. Additionally, the length of time and temperature for incubation of liver subfractions with [^{125}I]-AngII had to be optimised. Experiments confirmed incubation for 1 hour at room temperature (22°C) with 0.5 nM [^{125}I]-AngII provided optimum binding conditions.

Under these conditions, specific binding of [^{125}I]-AngII was measured in liver homogenates. Specific binding was detected with a K_D of 0.69 ± 0.3 nM. This binding affinity is similar to that seen in human heart (Nozawa et al. 1994) and greater than values reported in the literature for whole rat liver homogenates (Karamyan et al. 2009) suggesting appropriate conditions to perform further experiments.

5.3. Specific binding of [^{125}I]-AngII in crude mitochondria from rat liver

	K_D (nM)	B_{MAX} (fmol/mg)
Rat 1	0.43 ± 0.41	6.82 ± 4.3
Rat 2	0.51 ± 0.49	4.60 ± 3.6
Rat 3	0.34 ± 0.32	3.73 ± 2.8
Pooled data n=3	0.59 ± 0.33	5.05 ± 1.1

Table 16. Specific binding of [^{125}I]-AngII in crude mitochondrial fractions isolated from livers of 12-week old male Sprague Dawley rats. Data represent mean ± SD.

These data confirm binding of [^{125}I]-AngII in rat liver crude mitochondrial fractions with a specific binding affinity in the same range as AngII binding at the plasma membrane and with a receptor density consistent with functional G protein-coupled membrane receptors (Karamyan et al. 2009; Nozawa et al. 1994).

5.4. Specific binding of [^{125}I]-AngII in pure mitochondria and MAM

In order to measure specific binding in pure mitochondria and MAM, combined subfractions from 5 individual rats were required for each assay. Data presented represent three independent experiments each with pooled (x 5) membranes.

Pooled data (n=3)	K_D (nM)	B_{MAX} (fmol/mg)	nH
PM	0.38 ± 0.30	0.42 ± 0.06	0.90 ± 0.17
MAM	0.28 ± 0.12	15.34 ± 5.1	1.01 ± 0.03

Table 17. [^{125}I]-AngII saturation binding in pure mitochondria and MAM. Pooled (x5) mitochondrial membrane preparations for MAM and PM were used to measure saturation binding. Assays were performed in triplicate. PM; pure mitochondria, MAM; mitochondria associated membranes. Data represent mean ± SD.

Specific binding of [^{125}I]-AngII with high affinity was observed in both pure mitochondria and MAM. The Hill coefficient close to 1 is indicative of either a single population of AngII receptors or multiple populations of receptors with similar affinity for [^{125}I]-AngII. Since AngII shows 15 fold selectivity for AT_2R over AT_1R (Bosnyak et al. 2011), these data would favour the presence of a single AngII receptor type.

Though the B_{MAX} for [^{125}I]-AngII in MAM is high, that for the specific binding in pure mitochondria is below that usually considered consistent with a functional receptor system. The most likely explanation for this is contamination of pure mitochondrial fractions with MAM.

5.5. Identification of AngII receptor type in pure mitochondria and MAM

Having determined the K_D for [^{125}I]-AngII in crude mitochondrial subfractions, competition binding assays were performed using specific antagonists at the AT_1R (losartan) and AT_2R (PD123319).

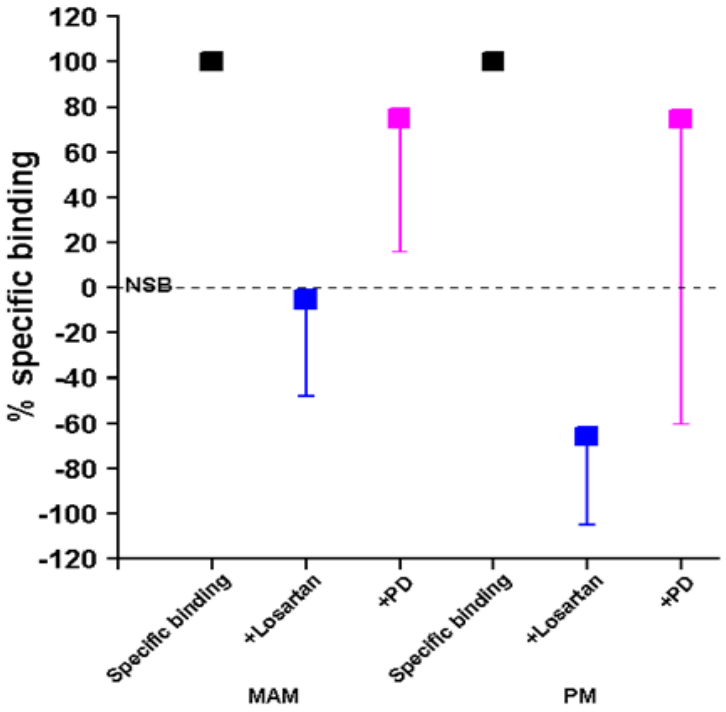

Figure 25. [^{125}I]-AngII specific binding in MAM and pure mitochondrial fractions in the presence of AT_1R and AT_2R antagonists. MAM or pure mitochondria (PM) were incubated with [^{125}I]-AngII for 1 hour at room temperature (22 ˚C), with or without the addition of Angiotensin-II (10 μM) to determine non-specific binding. Additionally, either losartan (1 μM) or PD123319 (1 μM) were included to determine presence of AT_1R vs. AT_2R receptors. Data are presented as percentage of specific binding in each fraction. Data are plotted as mean ± SD.

In the MAM, specific binding was completely inhibited by incubation with the AT_1R receptor antagonist losartan whilst PD123319 did not significantly reduce binding. Similar findings were observed in the pure mitochondrial fraction.

5.6. Conclusions

These data show that in rat hepatocytes, radiolabelled AngII binds specifically and with high affinity to receptors in the MAM and pure mitochondria, but only in the MAM is the density of receptors compatible with a functioning receptor system. Further, these receptors are likely AT_1R since the Hill coefficient suggested a single receptor subtype and binding was completely inhibited by the AT_1R selective inhibitor, losartan. The binding in pure mitochondria is likely a consequence of contamination of the PM fraction with MAM membranes, supported by the data presented in Table 5 (p.82), suggesting contamination is inherent to the isolation protocol.

These findings contradict other published reports. Of the studies identifying mitochondrial [^{125}I]-AngII binding, Sirett studied brain tissue whilst van Kats used renal and adrenal tissue; the possibility that mitochondrial AngII receptors exist in these tissues cannot be excluded. However, of note van Kats used a crude mitochondrial fraction to measure mitochondrial binding (therefore consistent with the data presented in this chapter) and noted significant lysosomal contamination (van Kats et al. 2001). In Sirett's study, the B_{MAX} of [^{125}I]-AngII binding in the mitochondrial fraction was 1.28 fmol/mg suggesting very sparsely distributed receptors, again raising the possibility of contamination (Nancy E. Sirett et al. 1977). Importantly in reference to the findings of Abadir *et al* (Abadir et al. 2011), these radioligand binding studies provide no evidence for AT_2R in mitochondria.

There are limitations to ligand binding assays which should be considered. These assays were carried out under non-physiological conditions (room temperature) so may not accurately represent pharmacokinetics *in vivo*. Additionally the intracellular environment contains endopeptidases which metabolise AngII. This has two consequences for radioligand binding studies. In homogenates, [^{125}I]-AngII is metabolically degraded to [^{125}I]-AngIII which also binds AT_1R and AT_2R making

accurate measurement of [^{125}I]-AngII binding difficult (Karamyan et al. 2009). Conversely, in mitochondrial isolates (lacking cytoplasm) the usual *in vivo* degradation does not occur making it an inaccurate representation of cellular physiology. It cannot be excluded that the described effects of AngII on mitochondria within cellular environments are due to angiotensin fragments. However, it is unlikely that a complete mitochondrial RAS would operate independent of AngII action at a receptor, making this possibility unlikely.

A further consideration is that it remains unclear whether [^{125}I]-AngII is able to cross the mitochondrial membrane. As such, if an AngII binding site existed within the inner mitochondrial membrane or matrix, this may not bind radioligand causing a false negative result. To explore this possibility, further experiments could be performed using inner mitochondrial membrane preparations pooled from a large number of livers (approximately 20 per assay).

The presence of AT_1R in MAM fractions in high density requires explanation. Two scenarios seem most likely; they represent receptors being trafficked to or from the plasma membrane or other intracellular organelles via the MAM, or they are functioning receptors for AngII in a MAM-mitochondrion microdomain.

With regards the former, the general model of G protein-coupled receptor trafficking involves initial transport to the ER where they undergo processing and folding (Drake et al. 2006). Given MAM is ER membrane that is transiently and dynamically associated with mitochondria (Bravo et al. 2011; Rizzuto et al. 1998; Hamasaki et al. 2013), the AT_1R present in these studies may represent receptors being trafficked through the ER.

The counter-argument is that MAM AT_1R are present by design rather than coincidence. It seems unlikely that such high density of receptors would be found if they were solely being trafficked, whilst it is accepted that the proteome of the MAM

is regulated independent of the rest of the ER allowing for such concentration of proteins. Evidence to support this hypothesis would be provided by performing [^{125}I]-AngII radioligand binding studies with (non-mitochondrial associated) ER and compare receptor density to that found in the MAM; if significantly lower in the ER it would suggest programmed accumulation in the MAM.

The possibility remains, therefore, that MAM AT_1Rs are part of a functional MAM-mitochondrial RAS. Direct interaction of MAM receptors and proteins of the mitochondrial OMM have been well described (Szabadkai et al. 2006; Csordás et al. 2006). Even if a direct communication between AT_1R and OMM is not apparent, given the range of signalling cascades that the AT_1R induces upon activation, it is conceivable that second messengers may effect a crosstalk with mitochondria. The IP_3 - IP_3R - Ca^{2+} - VDAC signalling system would provide such a link. The MAM-mitochondrial Ca^{2+} signalling pathway is a target of many signalling cascades (Hayashi & Su 2007) and is largely orchestrated through the IP_3R. Since activation of AT_1R is known to induce IP_3 release (Montezano et al. 2014) activation of such a system is plausible. Given the known effects of Ca^{2+} in modulating mitochondrial bioenergetics and ROS production (see Introduction, Chapter 1.9, p.40), activation of MAM AT_1R could allow the rapid tuning of mitochondrial metabolism. Assessment of alterations in mitochondrial respiration in the presence of AngII was therefore carried out.

6. RESULTS:

EFFECT OF ANGII ON RESPIRATION OF ISOLATED LIVER MITOCHONDRIA

6.1. Introduction

Published data convincingly support an effect of AngII on aspects of mitochondrial physiology (see Introduction Chapter 1.10, p.41). However, most studies have assessed mitochondria within their intact cellular environment, or mitochondria isolated from tissues following administration of AngII in animal models. Disentangling plasma receptor- or nuclear receptor- mediated effects from direct mitochondrial effects is not possible in these experimental designs.

Results of the radioligand binding studies taken together with the mass spectroscopy data make conventional action of AngII on the AT_1R or AT_2R at the mitochondrion unlikely. However, the possibility of AngII acting directly in a non-canonical fashion remained a possibility. A direct effect on ETS complex function, membrane permeability (LEAK state) or intra mitochondrial metabolic pathways (such as the TCA cycle or β oxidation) could result in functional modulation.

Such effects should be evident in oxygen consumption studies with isolated mitochondria exposed to AngII. Of note, Abadir *et al* showed an overall decrease in State 3 respiration in isolated rat liver mitochondria in response to the synthetic AT_2R agonist CGP421140, an effect they suggested was linked to an increase in mitochondrial NO production (Abadir et al. 2011).

To investigate whether such effects could be shown using the endogenous agonist (AngII), crude mitochondria were isolated from 12-week old male Sprague Dawley rats and their oxygen consumption measured under various experimental conditions. Pure mitochondria were not used in these experiments since Percoll density separation by ultracentrifugation renders mitochondria dysfunctional whilst only crude mitochondria would allow for investigation of MAM-mitochondrial interactions (data not shown).

6.2. Effect of AngII on 'resting' isolated liver mitochondria

To investigate the effect of AngII on basal respiration, O_2 consumption of isolated mitochondria was measured in the absence (control) and presence of 1 µM AngII with no additional substrates or ADP. Measurements were taken at baseline and 5, 10, 15, 20 and 25 minutes following the addition of AngII or vehicle (water).

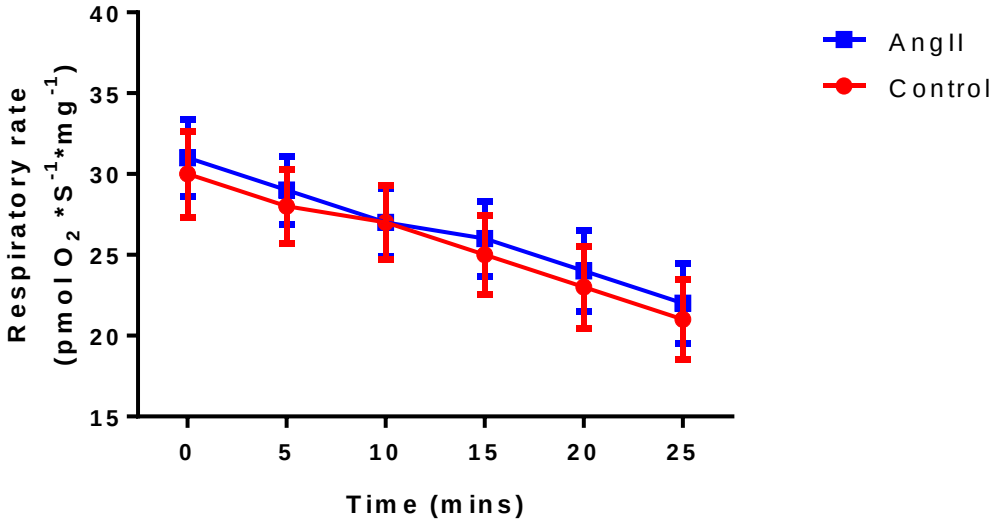

Figure 26. Basal respiration rates in isolated mitochondria with and without (control) 1 µM AngII. Data plotted as means ± SEM (n = 9). Differences along time between samples were assessed by ANOVA for repeated measures (time-group interaction: p >0.05).

There was no effect of 1µM AngII on respiration of unstimulated isolated liver mitochondria over the course of a 25 minute incubation. In the absence of exogenous substrates or ADP, mitochondria respire on endogenous substrates with ATP cycling by extra-mitochondrial ATPase activity. This is reflected in the decrease in respiratory rate observed over time, implying consumption of available substrates.

However, the ongoing (though low) rate of respiration at 25 minutes suggests a residual O_2 consumption. If it assumed all endogenous substrates have been consumed by this point, this represents mitochondrial non-ETS O_2 utilisation in side reactions that result in superoxide anion radicals and other reactive oxygen species (Cadenas & Davies 2000). From these data it can be concluded that AngII does not

increase this non-ETS O_2 utilisation, and therefore suggests in the resting state, AngII is unlikely to increase mitochondrial ROS production.

6.3. Effect of AngII on OXPHOS in isolated liver mitochondria

Since no effect of AngII on unstimulated mitochondria could be identified, an effect on actively respiring mitochondria was sought. Mitochondria were incubated in the respiration chamber at 37 ˚C for 5 minutes in the presence of NADH linked substrates (pyruvate 5mM and malate 5mM) with or without (control) AngII (in concentrations of 0.1 µM or 1 µM). After 5 minutes, respiratory States were measured as described in Methods Chapter 2.8.4.

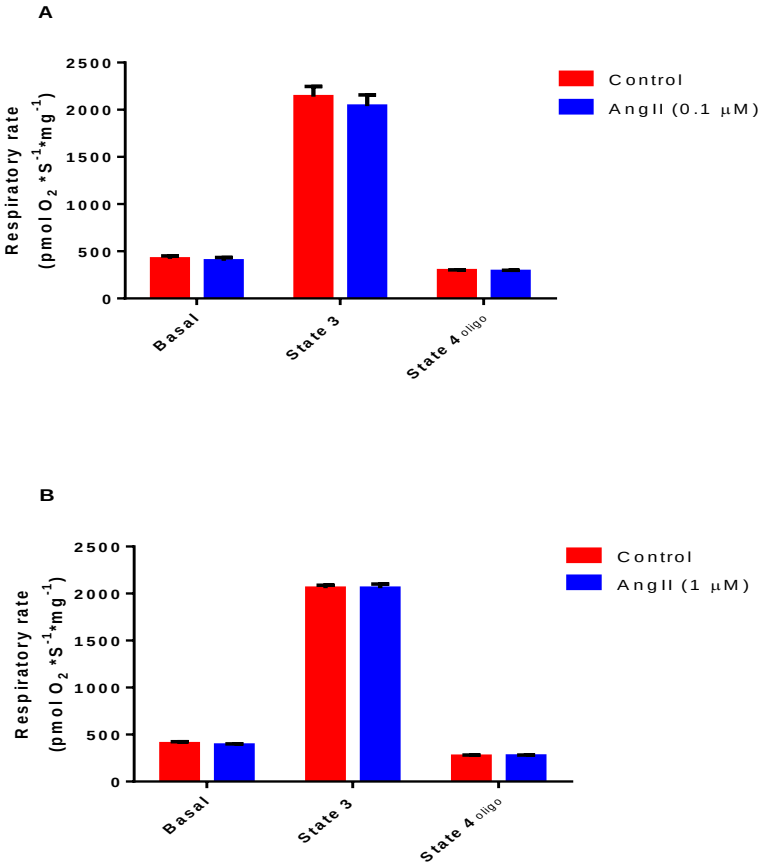

Figure 27. Respiration rates of isolated liver mitochondria in the presence of malate and pyruvate. Data are taken from three independent preparations (n=3). There was no significant effect of AngII on basal, State 3 or State 4 respiration at either 0.1 µM (A) or 1 µM (B). Data represent mean ± SEM and differences between AngII and control groups were assessed using paired sample t-tests.

	ADP/O	RCR
Control	3.20 ± 0.19	7.26 ± 0.40
AngII (0.1 μM)	3.23 ± 0.30 (NS)	7.13 ± 0.28 (NS)
AngII (1 μM)	3.21 ± 0.46 (NS)	7.58 ± 0.42 (NS)

Table 18. Coupling and efficiency of OXPHOS in mitochondria exposed to AngII. Using State 4_{oligo} to calculate RCR (RCR_{oligo}), there was no significant effect of AngII on RCR or ADP/O ratio. Data represent mean ± SEM. Differences vs. control were assessed using paired sample t-tests. NS; non-significant.

These experiments do not provide any evidence for an effect of AngII on mitochondrial respiration. The absolute rates of respiration (basal, State 3 and State 4_{oligo}) were unchanged in the presence of AngII at 0.1 μM and 1 μM. State 3 respiration reflects the activity of ATP turnover (encompassing adenine nucleotide translocase activity, phosphate transportation and ATP synthase) and substrate oxidation (involving substrate uptake, TCA enzyme activity, ETS complex activities, the available pool of ubiquinone and cytochrome C, and $[O_2]$). An unaltered State 3 implies that theses aspects of OXPHOS function are not affected by AngII. Meanwhile, State 4_{oligo} predominantly reflects proton leak and is therefore a good measure of membrane integrity, LEAK, proton slip and uncoupling. A lack of difference in State 4_{oligo} respiration rates suggests that AngII does not modulate proton leak. These conclusions are supported by the maintained RCR in AngII treated mitochondria.

The ADP/O ratio is often used as a measure of mitochondrial OXPHOS 'efficiency' (see p.72). Change in ADP/O predominantly reflects alterations in the coupling mechanism itself i.e. proton slip (at complexes I, III and IV). The lack of change in ADP/O ratios in the presence of AngII therefore suggests no direct effect on respiratory complex efficiencies.

These data do not, however, entirely rule out an effect of AngII on mitochondrial respiration. Whilst the absolute respiratory rates and RCR provide a relatively sensitive overview of mitochondrial respiration, they may not identify more subtle defects of respiration at individual complexes of the ETS. In reporting respiration with malate and pyruvate as substrates, complex II function cannot be commented on. Complex II is not involved in respiration on malate/pyruvate in isolated mitochondria (Fig. 28). In the presence of high malate concentrations, fumarate levels quickly equilibrate, inhibiting succinate reduction at succinate dehydrogenase (complex II) and therefore preventing $FADH_2$.formation. Additionally, due to the high activity of the tricarboxylate carrier in liver mitochondria, in the presence of high malate concentration, citrate is lost to the cytoplasm (or respiration medium) limiting forward flux to succinate (Gnaiger 2007).

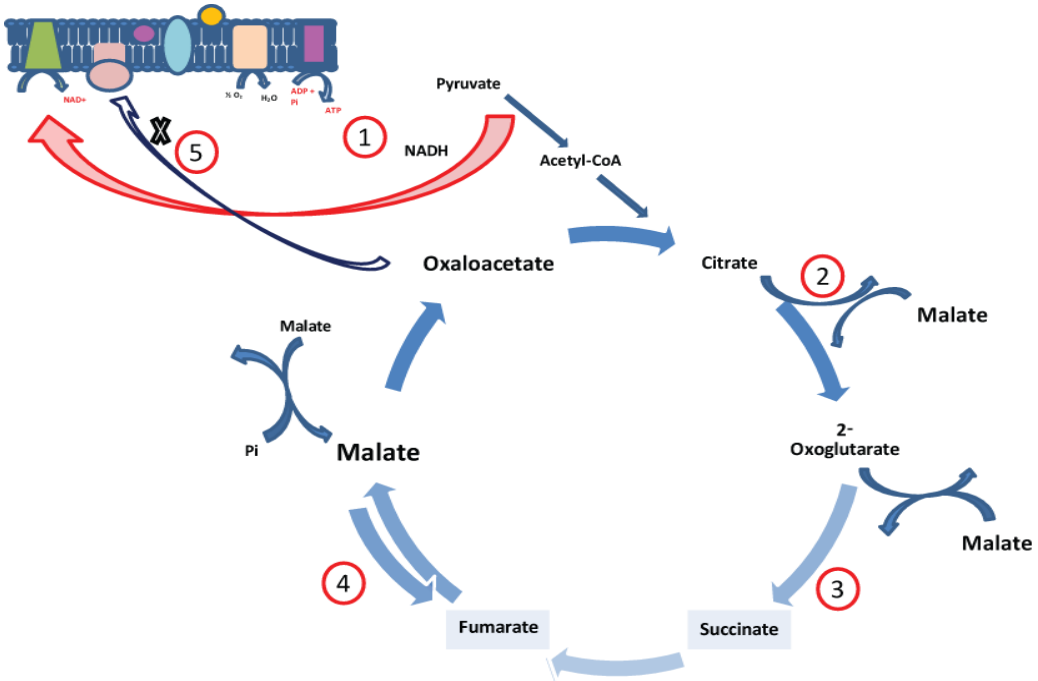

Figure 28. Metabolism of pyruvate and malate in insolated liver mitochondria. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase forming NADH which supplies electrons to complex I (1), whilst acetyl-CoA enters the TCA cycle forming citrate from combination with oxaloacetate. In the presence of high malate concentrations, citrate is lost to the matrix in exchange for malate influx via the tricarboxylate carrier (2) with subsequent decreased succinate formation (3). High malate concentrations also equilibrate with fumarate thus decreasing forward flux from succinate to fumarate (4). There is also a direct inhibitory effect of oxaloacetate (increased in this context) on succinate dehydrogenase (5).

Further, the AngII concentrations applied in these experiments may have been suboptimal in timing and dose. A dose dependent effect of AngII in renal proximal tubule transport has been described; picomolar AngII concentrations have a stimulatory effect and a nanomolar - macromolar concentration causes inhibition (Shirai et al. 2014). Therefore, further experiments were conducted using a broader range of AngII concentrations, whilst interrogating individual complexes of the ETS.

6.4. Effect of AngII on individual complexes of the ETC in isolated mitochondria

To investigate the effect of AngII on individual complexes of the respiratory chain, high resolution respirometry was performed with isolated mitochondria. These experiments were conducted using the Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria). Substrate-uncoupler-inhibitor titration (SUIT) protocols were used to isolate individual complexes of the ETS and measure respiration rates in State 3 and State 4. RCR was also calculated. Doses of AngII used were extended to range from 1 nM to 1000 nM.

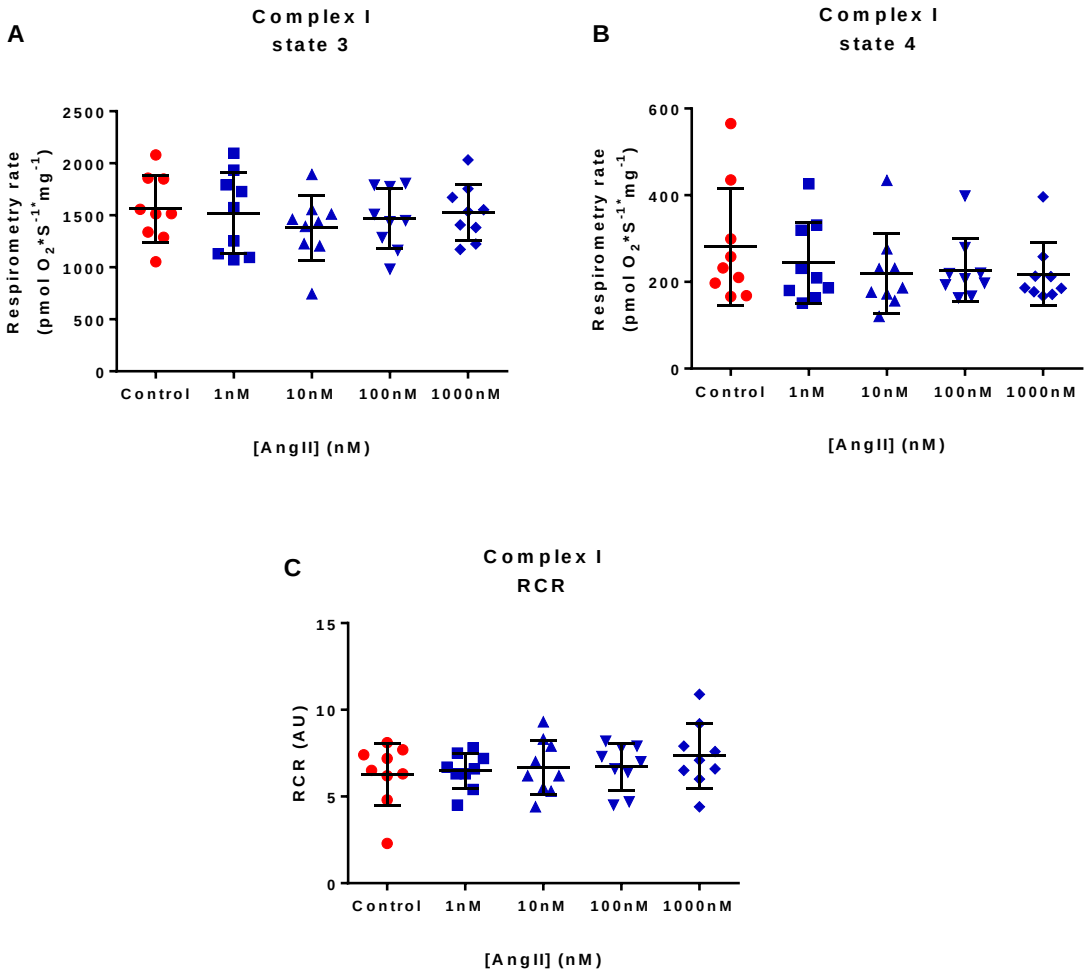

Figure 29. Respiration rates at complex I with and without (control) AngII. State 3 respiration was measured in the presence of malate (5 mM) and glutamate (10 mM) after addition of ADP (0.5 mM). Data presented as dots (n=9) with mean and 95% confidence intervals marked. Difference between treatment and control samples was assessed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. There were no significant effects of AngII at 1, 10, 100 or 1000 nM concentrations on State 3 or State 4 respiration. There were no significant differences in RCR ($p < 0.05$ considered significant for AngII vs. controls).

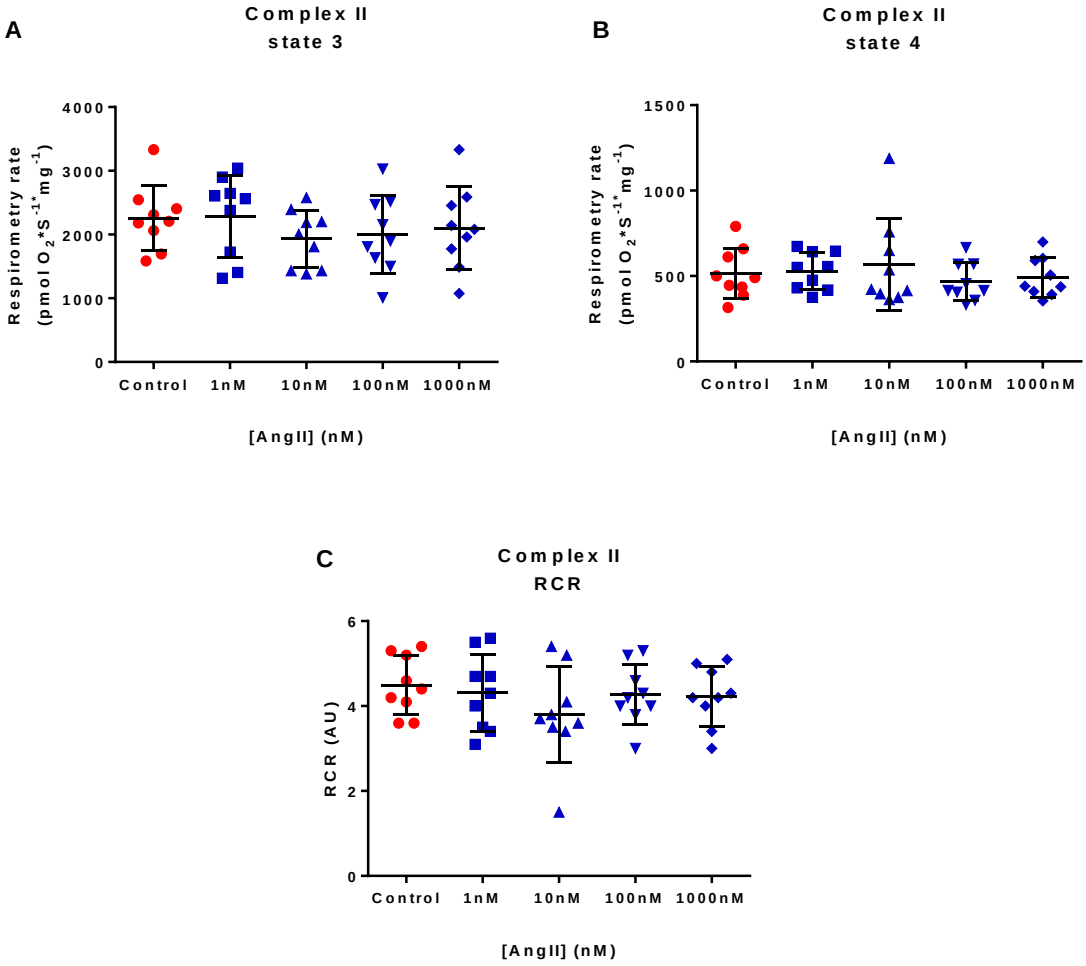

Figure 30. Respiration rates at complex II with and without (control) AngII. State 3 respiration was measured in the presence of rotenone (0.5 μM) and succinate (10 mM) after addition of ADP (0.5 mM). Data presented as dots (n=9) with mean and 95% confidence intervals marked. Difference between treatment and control samples was assessed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. There were no significant effects of AngII at 1, 10, 100 or 1000 nM concentrations on State 3 or State 4 respiration. There were no significant differences in RCR ($p < 0.05$ considered significant for AngII vs. controls).

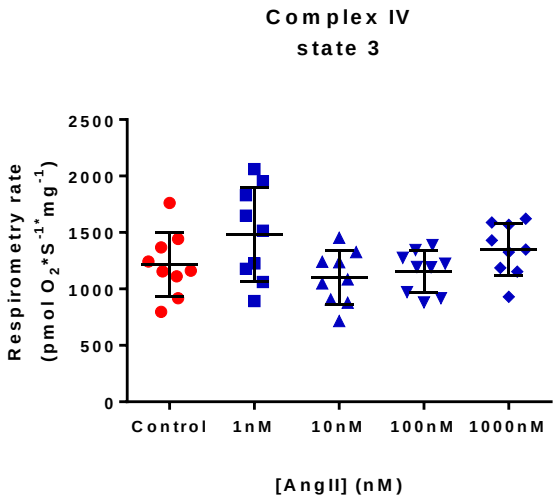

Figure 31. Respiration rates at complex IV with and without (control) AngII. State 3 respiration was measured in the presence of ascorbate and TMPD after addition of ADP (0.5mM). Values were corrected for auto-oxidation of substrates by addition of sodium azide. Data presented as dots (n=9) with mean and 95% confidence intervals marked. Difference between treatment and control samples was assessed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. There were no significant effects of AngII at 1, 10, 100 or 1000 nM concentrations on State 3 respiration ($p < 0.05$ considered significant for AngII vs. controls).

These graphs show that presence of AngII in concentrations ranging from 1 nM to 1 µM has no significant effect on respiration at complex I, II or IV.

6.5. Effect of acute addition of AngII on ETS complex function

Although no significant effects on mitochondrial respiration were seen in response to incubation with AngII, it is conceivable that AngII has a short lasting action on mitochondria, occurring within a shorter time frame. This is supported by estimates of the half-life of AngII in the circulation as approximating 16 ± 1 seconds (Al-Merani et al. 1978).

To investigate whether there was a short-lived effect of AngII on mitochondrial respiration, experiments were conducted in which AngII was added to mitochondria respiring on complex I or complex II substrates in the presence of ADP (State 3). A difference in respiration rates before and after the addition of AngII was sought.

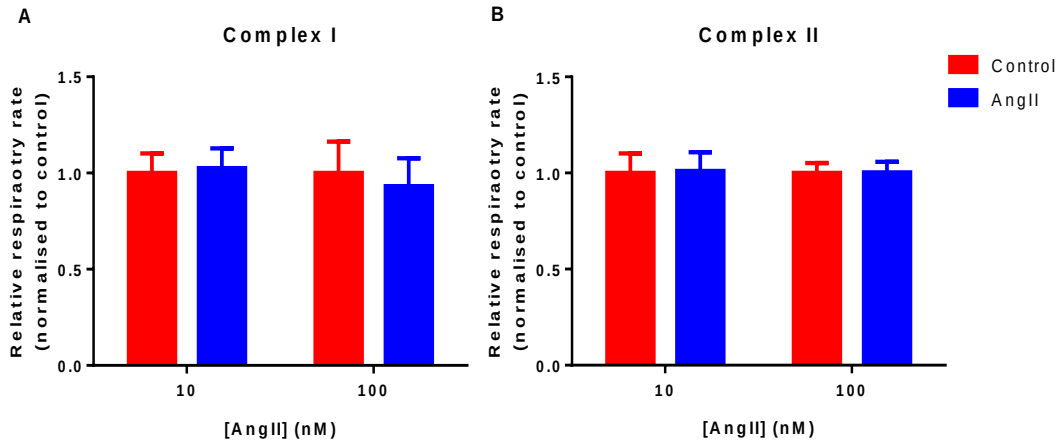

Figure 32. Effect of acute addition of AngII on State 3 respiration at (A) complex I and (B) complex II. No significant effect was observed with acute addition of AngII at either 10 nM or 100 nM at complex I or complex II. Data represent mean ± SEM. Differences were assessed using paired sample t-tests.

As shown in Figure 33, acute addition of AngII at either 10 nM or 100 nM did not have any significant effect on State 3 respiration at either complex I or complex II.

6.6. Conclusions

In these experiments, an effect of AngII on mitochondrial respiration was sought by measuring oxygen consumption rates of isolated liver mitochondria. Incubation of resting mitochondria (in the absence of exogenous substrates or ADP) with 1 µM AngII caused no significant change in respiration suggesting that there is no effect of AngII on mitochondrial inner membrane integrity or basal proton leak in the resting (unstimulated) state.

An effect of AngII on 'actively' respiring mitochondria was then sought. As a primary survey of overall mitochondrial respiration, isolated liver mitochondria were incubated with NADH linked substrates and respiratory capacity was assessed. Following a 5 minute incubation with AngII (100 nM or 1000 nM) or placebo at 37 $^{\circ}$C, no effect on overall respiratory rates could be demonstrated, with RCR and ADP/O ratios unaltered.

Interrogation of individual complexes of the ETS failed to demonstrate any inhibitory effect of AngII on mitochondrial respiration at complexes I, II and IV in concentrations ranging from 1 nm to 1 µM. This represents [AngII] in physiological to supraphysiological levels. The concentration of circulating AngII in rats is in the picomolar range, whilst tissue levels range from picomolar to low nanomolar concentrations depending on organ (Kai et al. 1998; Liu et al. 2011; Nishiyama et al. 2003). Though intracellular concentrations have not been defined, the sub-nanomolar affinity of the AT_1R and AT_2R in the radioligand binding assays would suggest similarly low intracellular concentrations *in vivo*.

These data are in contradiction to data reported by Abadir *et al* who described decreased State 3 respiration in isolated liver mitochondria in response to the synthetic AT_2R agonist CGP421140 (Abadir et al. 2011). However, no effect was

detailed with AngII, and so the physiological relevance of such finding must be questioned.

The physiological relevance of these data has also to be questioned in other ways. Assessment of mitochondrial function using oxygen consumption of isolated mitochondria in a respirometry chamber is a well-established model. However, this *in vitro* setting differs from the *in vivo* environment on many counts. The O_2 tension applied in most protocols is higher than *in vivo*, a necessary measure in order to allow for oxygen consumption during experimental protocols. Substrate concentrations are supra-physiological, with [succinate] being particularly anomalous; the cellular physiological concentration of succinate in healthy cells is negligible (Shabalina & Nedergaard 2011). Isolating ETS complex function by supplying NADH linked substrates and FADH linked substrates is also unphysiological; *in vivo*, both complex I and complex II are active, with electron flow meeting at the Q junction. By assessing maximal respiration without accounting for flux through complex II, the protocols used may have underestimated State 3 respiration. Finally, the concept of respiratory states as defined by Chance and Williams are by design not physiological; it is unlikely that mitochondria are ever maximally respiring, nor in true State 4 (evidenced by the fact that oligomycin applied to most cell preparations will cause a decrease in the respiratory rate).

A particular disadvantage of working with isolated mitochondria is that they are removed from, and can no longer interact with, their cellular environment. This is important given the roles mitochondria are now appreciated to play in cell signalling pathways. With regards respiration, this results in accumulation of some TCA cycle intermediaries whilst others are consumed or lost to the respiratory medium when *in vivo* they would be cycled back from the cytoplasm (Rasmussen et al. 2001).

However, accepting these limitations, the lack of effect of AngII (in the physiological range) on isolated mitochondrial respiration strongly suggests absence of a functioning mitochondrial RAS or any direct effect of intracrine AngII on the organelle in rat liver.

7. DISCUSSION

7.1. Summary of findings

Given the weight of evidence in the literature supporting an effect of RAS on cellular energetics, of an intracellular site of action for AngII and of the pivotal role that mitochondria play in regulating cellular bioenergetic state, the possibility of a mitochondrial RAS orchestrating aspects of mitochondrial function was an attractive hypothesis. However, the data presented in this thesis provide no evidence for such a system, in rat liver mitochondria at least.

Analysing the proteome of the mitochondrial fractions isolated by sequential centrifugation of rat liver homogenates did not identify any integral member of the RAS family. The presence of neurolysin in the pure mitochondrial fraction warranted discussion given its identification as a novel AngII binding protein in rat brain homogenates *in vitro*. However, there is no evidence that neurolysin binds AngII in liver, and no evidence for the AngII binding capacity of neurolysin *in vivo*.

Given that the pure mitochondrial fraction only contained 27.8% of known mitochondrial proteins, a survey of existing mitochondrial proteome databases was conducted. None of the recognised first-order RAS proteins were found; in particular no AngII receptor was present. Whilst these data made the existence of a liver mitochondrial RAS less likely, they could not completely refute the hypothesis since proteins expressed in low abundance may not be recognised in a general proteomic screen.

Thus, immunochemistry was employed to identify mitochondrial RAS proteins. Accepting that enzymes and effector proteins of the RAS may only be fleetingly associated with mitochondria, efforts concentrated on identifying an AngII receptor (namely AT_1R and AT_2R). With immunoreactive bands identified at the appropriate weights for both receptors, immunoprecipitation was employed to increase the protein yield and, therefore, the sensitivity of mass spectrometry. Mass

spectrometry failed to identify any AngII receptor peptides. However, this work was undermined by the non-specific nature of the primary antibodies employed. For the same reason, the results of Western blotting using these antibodies have to be ignored.

In the absence of specific antibodies, a pharmacological approach was taken to identify possible mitochondrial AngII binding sites. Employing the radioiodinated version of the major physiological RAS ligand (^{125}I-AngII), specific binding to a single receptor population was demonstrated in both the pure mitochondrial and MAM fractions. However, the low abundance of receptors in the pure mitochondria is not consistent with a functioning G protein-coupled receptor system, and likely the result of contamination with MAM. This is supported by the mass spectrometry data showing only 64.9% of the proteins in the pure mitochondrial fraction to be mitochondrial. The identity of the AngII binding site in the MAM was confirmed as AT_1R by competition binding assays.

Whether MAM AT_1Rs represent a functional mitochondrial-associated RAS system or whether AngII has a direct non-canonical action at the mitochondrion was investigated with respirometry assays. AngII had no effect on respiration in concentrations ranging from physiological to supra-physiological concentrations, making an *in vivo* action unlikely.

These data strongly suggest that a mitochondrial associated RAS does not exist in rat liver mitochondria. This is in contradiction to much of the literature cited in support of this hypothesis. Reconciling these data requires close inspection of the published work.

7.2. Reconciling these findings with the literature

Much of the published data in support of a mitochondrial RAS is reliant in part, or entirely, on non-specific antibodies and must therefore be treated with caution. In

the study by Benicky *et al*, all the commonly employed AT_1R antibodies were shown to be non-specific. Given the reliance of Western blotting, immunoprecipitation, immunocytochemistry and flow cytometry on antibody specificity, the validity of a large body of published work must be questioned. Indeed, up to March 2012, 3,360 publications were listed on PubMed under the search term 'Angiotensin II AT_1 receptor antibodies', whilst 338 results were reported under the term 'AT_1 receptor western blotting' (Benicky et al. 2012). A similar story is apparent with regards AT_2R antibodies (Hafko et al. 2013).

With this in mind, re-examination of the data cited in support of my hypothesis leads to a less convincing story. Abadir *et al* argue for the presence of functional AT_2R in mitochondria from rat heart and liver in direct contradiction with my data. However, in the immunogold and immunofluorescence labelling studies they employed a Santa Cruz AT_2R antibody (Sc-9040) now known to be non-specific. The work by Huang *et al* is often cited as evidence for mitochondrial AT_1R (Huang et al. 2003). However, the immunolabelling pattern in this study actually showed binding in the ER *near* mitochondria (likely the MAM), but not within mitochondria. There are also questions regarding the specificity of the antibody they raised against the carboxyl terminal of AT_1R.

Erdmann *et al* demonstrated immunolabelled AngII within mitochondria of rat adrenal, liver and brain tissue (Erdmann et al. 1996). However, evidence of specificity for the antibody against AngII was not provided and the authors admit a significant proportion of this binding was non-specific.

In cells of the adrenal cortex and zona glomerulosa, Peters *et al* demonstrated 'dense bodies' containing renin (with or without angiotensinogen) in the mitochondrial fraction on subcellular fractionation (Peters et al. 1996). However, using the same technique, Mizuno showed the dense bodies to be distinct from

mitochondria (Mizuno et al. 1991). Again, evidence of specificity for the antibodies employed is not provided in this or associated work (Wanka et al. 2009; Inagami et al. 1980).

Of the work not relying on specific antibodies, Clausmeyer *et al* reported a mitochondrially targeted renin which could be imported across the membrane (Clausmeyer et al. 1999). Whilst the evidence for an alternative renin transcript lacking the secretary signal is convincing in cardiac and adrenal tissue at least, (Clausmeyer et al. 2000) its import into mitochondria has not been demonstrated. It has been shown that a truncated prorenin construct can be imported across the mitochondrial membrane but this construct differs in structure from the identified cytosolic renin.

What of the literature involving radioligand binding studies? Van Kats reported [^{125}I]-AngII binding in the mitochondrial fraction of both renal and adrenal homogenates (van Kats et al. 2001). However, these binding studies were conducted with a crude mitochondrial fraction, with evidence in their paper of significant contamination of that fraction with lysosomes and microsomes.

The more convincing evidence is that of mitochondrial [^{125}I]-AngII binding in rat brain homogenates (Nancy E. Sirett et al. 1977). Whilst the majority of binding occurred in the microsomal fraction, and some of the binding may have been non-specific, it does seem that binding occurred in the pure mitochondrial fraction. This suggests that [^{125}I]-AngII binding differs in rat liver and brain. Given the *in vitro* AngII binding property of mitochondrial neurolysin differs in brain and liver this perhaps should not surprise. Additionally, some caution is necessary when translating these finding to *in vivo* settings; binding was shown only in the presence of EDTA and as such, in a non-physiological environment.

Finally, the evidence for AngII trafficking to the mitochondria of cardiac muscle provided by Robertson *et al* (Robertson & Khairallah 1971) is limited to a single comment in text without any supporting images or data and is therefore difficult to evaluate.

7.3. Do MAM AT_1R represent a mitochondrial-associated RAS?

The AT_1R identified in the MAM by radioligand binding studies are in a high enough density to be compatible with a functional G-protein linked receptor signalling system. It is now widely appreciated that signalling at the MAM-mitochondrial interface can influence many aspects of mitochondrial function including respiration (see Introduction section 1.9, pp.39-40). However, in respirometry studies conducted with crude mitochondria (and therefore including MAM), no physiologically relevant functional effect of AngII could be demonstrated. This can be explained in two ways. In the first scenario, the AT_1R in the MAM are bystanders; present only as a consequence of their passing through the ER on the way to or from the plasma membrane or nucleus. In support of this, a trafficking pathway for plasma membrane AT_1R via the Golgi apparatus and ER has been described (Hunyady et al. 2002; Bkaily et al. 2003; Cook & Re 2009) whilst it is known that the portion of the ER forming the MAM is fluid with both mitochondrial and MAM surfaces in constant flux (Grimm 2012).

In the second scenario, the MAM AT_1R do influence mitochondrial function *in vivo*, but this system is rendered dysfunctional by the process of *ex vivo* isolation. The proximity of MAM to the outer mitochondrial membrane (OMM) seems to be vital for efficient signalling (Csordás et al. 2006; Csordás & Hajnóczky 2009). Although the tethering between OMM and MAM is tight enough to withstand centrifugation at 10,000 g, the distance between membranes in crude mitochondrial preparations may be altered, making the MAM signalling domain dysfunctional. Further, it is unclear whether MAM retains the capacity to release Ca^{2+} *in vitro,* which appears

integral to most MAM signalling processes. Additionally, the respiration medium includes the Ca^{2+} chelating agent EGTA which may limit ER Ca^{2+} accumulation.

One way to determine whether isolated crude mitochondria retain functional MAM-mitochondrial domains would be to measure mitochondrial response to MAM receptor stimulation. With the very recent advancements in high resolution respirometry allowing measurement of mitochondrial membrane potential and [Ca^{2+}] in the chamber (Fasching & Gnaiger 2009), MAM IP_3R stimulation using IP_3 may provide an answer to this question.

Functional imaging may provide an alternative method for interrogating MAM AT_1R signalling. Replicating experiments in which AngII is injected intracellularly in cells in which plasma membrane receptors have been blocked with losartan may provide a model whereby the mitochondrial response (Ca^{2+} accumulation or membrane depolarization) in the presence or absence of candidate MAM signalling cascade agonists might be measured.

7.4. Alternative mechanisms to explain mitochondrial effects of AngII

If a direct action of AngII on mitochondria does not exist, an alternative explanation is needed for the documented effects of AngII on mitochondrial physiology. Given the plethora of signalling pathways activated by AngII receptor binding there are many potential mechanisms. Of these, the role of NAD(P)H oxidases (Nox) seem worthy of particular inspection.

The AngII stimulated increase in mitochondrial ROS, decreased mitochondrial membrane potential and decreased RCR are all attenuated by inhibition of Nox (Doughan et al. 2008). Evidence also suggests that the stimulation of mitochondrial ROS by AT_1R requires the full activity of Nox and may depend on the activation of mitochondrial K_{ATP} channels (Dikalov & Nazarewicz 2013). Activation of plasma

membrane Nox by AT_1R is well described and Nox have since been shown to be expressed in renal nuclei where signalling appears to be via an AT_1R-PKC-PI3 dependent pathway (Seshiah et al. 2002; Pendergrass et al. 2009). Moreover, recent work suggests the presence of a Nox subtype (Nox4) in mitochondria of renal (Block et al. 2009) and cardiac cells (Kuroda et al. 2010; Ago et al. 2010) where it induces H_2O_2 production and reduces respiratory capacity, possibly by decreased complex I activity (Kozieł et al. 2013). Finally, numerous groups have produced work suggesting AngII induces increased Nox4 expression in mitochondria of various tissues (Lee et al. 2013; Kim et al. 2012; Horne et al. 2012; Case et al. 2013). Taken together these data suggest AngII may be operating through Nox4 to influence mitochondrial physiology.

However, although these data are intriguing, they are not conclusive. Other groups have failed to demonstrate mitochondrial Nox1, Nox2 or Nox4 (Doughan et al. 2008) whilst the specificities of the antibodies commonly employed have been questioned (Dikalov & Nazarewicz 2013). The concentration of AngII used in most studies is supra-physiological (1 µM) and no study has been able to demonstrate Nox4 *activity* in mitochondrial preparations. Furthermore, mitochondrial ROS production can be independent of Nox whilst the contribution of mito K_{ATP} to membrane potential is only approximately 1-2 mV implying that the changes in potential seen with AngII are more likely due to other mechanisms.

One such mechanism might depend on the activity of uncoupling proteins; the stimulation of mitochondrial ROS by AngII may be due to a decrease in the ability to uncouple OXPHOS. In support of this hypothesis, renal mitochondria of spontaneously hypertensive rats show increased UCP2 expression following AT_1R blockade whilst in an aging rat model both ACE inhibition and AT_1R blockade were associated with higher UCP2 concentrations in renal mitochondria (de Cavanagh et

al. 2003). The influence of AngII and other RAS components on UCP2 expression warrants further investigation.

7.5. Conclusion

My hypothesis stated that a mitochondrial RAS exists in liver mitochondria. These data do not support this hypothesis, and whilst it is difficult to conclusively prove the absence of a RAS, the weight of evidence is strongly against its existence. I cannot, however, exclude the presence of a mitochondrial RAS in other tissues, since mitochondrial biochemistry is heterogeneous between organs, and even within tissues. Further work in other organs is required to investigate this possibility. The mechanism(s) by which AngII influences mitochondrial physiology is still unclear and studies should concentrate on investigating other pathways, with Nox and UCP modulation foremost among them.

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APPENDIX

1:MATERIALS AND SOLUTIONS USED FOR MOLECULAR BIOLOGY

1.1. Isolation of mitochondria and other subcellular fractions

Reagents

AngII (1 µM stock) # 151039 M005, Alexis biochemicals, Exeter, UK

Ethylenediaminetetraacetic acid (EDTA): 100 mM

Phenylmethanesulfonyl fluoride (PMSF), 100nM

Protease inhibitor cocktail: Complete Mini, EDTA free, Roche, Herts, UK

Proteinase K: P6556, Sigma-Aldrich, MO, USA

Sodium carbonate, 100mM (pH 11.5)

TCA 72%

Solutions

Isolation medium A

Ion/Reagent	Molecular Weight	Final Concentration
Mannitol	182.17	250 mM
EGTA	380.4	0.5 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES	283.3	5 mM, pH 7.4

Isolation medium B

Ion/Reagent	Molecular Weight	Final Concentration
Mannitol	182.17	225 mM
EGTA	380.4	5 mM
HEPES	283.3	25 mM, pH 7.4

1.2. Protein quantification

Materials

Protease inhibitor cocktail	Complete Mini, EDTA free, Roche, Herts, UK
Phosphatase inhibitors	PhosStop, Roche, Herts, UK
BCA protein assay reagent kit	Pierce, Rockford, IL, USA

Solutions

Lysis buffer

Ion/Reagent	Molecular Weight	Final Concentration
Tris (pH 6.8)	121.3	100 mM, pH 6.8
NaCl	58	300 mM
nonyl phenoxypolyethoxylethanol - 40 (NP-40)	617	0.5%
H_2O	18	70%

1.3. Western blotting

Materials

Coomassie Brilliant Blue: R-250, Life Sciences, UK.

Primary antibodies
As listed in results section

Secondary antibodies

Antibody	Company	Concentration
Rabbit Anti-Goat IgG HRP conjugate	Invitrogen (Carlsbad, CA, USA)	1:4000
Donkey Anti-mouse IgG HRP conjugate	Invitrogen (Carlsbad, CA, USA)	1:4000
Goat Anti-rabbit IgG HRP conjugate	Invitrogen (Carlsbad, CA, USA)	1:4000

Solutions

Laemmli buffer

Ion/Reagent	Molecular Weight (g mol^{-1})	Final Concentration
Tris (pH 6.8) in 80% water/20% glycerol	121.3	0.125 M
Sodium dodecyl sulphate (SDS)	288.4	4%
2-mercaptoethanol	-	10%
Bromophenol blue	-	0.004%

Running buffer

20 x MOPS (diluted x20 with H_2O before use)

Ion/Reagent	Molecular Weight ($g\ mol^{-1}$)	Final Concentration
MOPS	209.3	1 M
Tris	121.1	1 M
SDS	288.4	2 %
EDTA	292.3	10 mM

Transfer buffer

Ion/Reagent	Molecular Weight ($g\ mol^{-1}$)	Final Concentration
Bicine	163.2	25 mM
Bis Tris	209.3	25 mM
EDTA	292.3	1 mM

Phosphate buffered saline Tween 20 (PBST)

Ion/Reagent	Final Concentration
Tween 20	0.5% (diluted in PBS)

Coomassie Brilliant Blue stain

Ion/Reagent	Final Concentration
Acetic acid	7%
Ethanol	40%
Coomassie Brillaint Blue	0.1%

1.4. Radioligand binding studies

Materials

Losartan # 3798, Torcis Bioscience, Bristol, UK

PD123319 # 1361, Torcis Bioscience, Bristol, UK

[^{125}I]Tyr^{4}-AngII (2200 Ci/mmol, 81.4 TBq/mmol): PerkinElmer, Cambridge, UK

Solutions

Radioligand assay buffer

Ion/Reagent	Molecular Weight (g mol^{-1})	Final Concentration
NaH_2PO_4	268	50 mM (pH 7.4)
NaCl	58	100 mM
EGTA	380.4	1 mM
$MgCl_2$	95.2	10 mM
BSA	66.5	0.2% (pH 7.4)

1.5. Respirometry

Materials

Antimycin A	A8574, Sigma Aldrich, MO, USA
AngII (1 µM stock)	# 151039 M005, Alexis biochemicals, Exeter, UK
Ascorbate	A4034, Sigma Aldrich, MO, USA
Glutamate	G1626, Sigma Aldrich, MO, USA
Losartan	# 3798, Torcis Bioscience, Bristol, UK
Malate	M9546, Sigma Aldrich, MO, USA
Oligomycin	75351, Sigma-Aldrich, MO, USA
PD123319	# 3798, Torcis Bioscience, Bristol, UK
Pyruvate	P8547, Sigma Aldrich, MO, USA
Rotenone	R8875, Sigma-Aldrich, MO, USA
Sodium Azide	S2002 Sigma-Aldrich, MO, USA
Succinate	S3674, Sigma-Aldrich, MO, USA
TMPD	T3134, Sigma-Aldrich, MO, USA

Solutions

Respiration medium

Ion/Reagent	Molecular Weight (g mol^{-1})	Final Concentration
Sucrose	342.3	110 mM
$MgCl_2$	95.2	3.0 mM
EGTA	380.4	0.5 mM
KCl	74.55	80 mM
K-lactobionate	358.3	60 mM
KH_2PO_4,	136	10 mM
Taurine	125.2	20 mM
HEPES	238.3	20 mM
BSA	66.5	1.0 gL^{-1} , pH 7.1

APPENDIX 2:

RAS GENE SEARCH TERMS USED IN PROTEOMICS ANALYSIS

Generation of RAS search terms

Gene ontology (GO) terms relating to the RAS were found using the AmiGO database (http://amigo.geneontology.org/amigo). The corresponding gene product names were identified using UniProt (http://uniprot.org/). The list detailed below was then compared to exiting mitochondrial databases (MitoCarta, MitoMiner) or reports of proteins identified by mass spectrometry of the PM, CM or MAM.

Entry	Entry name	Protein names	Gene names
Q5EGZ1	ACE2_RAT	Angiotensin-converting enzyme 2(ACE-related carboxypeptidase)	Ace2
P47820	ACE_RAT	Angiotensin-converting enzyme (ACE) [Cleaved into: Angiotensin-converting enzyme, soluble form]	Ace Dcp1
P35351	AGTR2_RAT	Type-2 angiotensin II receptor (Angiotensin II type-2 receptor) (AT2)	Agtr2
P25095	AGTRA_RAT	Type-1A angiotensin II receptor (Angiotensin II type-1A receptor) (AT1A)	Agtr1 Agtr1a At1a
P29089	AGTRB_RAT	Type-1B angiotensin II receptor (AT3) (Angiotensin II type-1B receptor) (AT1B)	Agtr1b Agtr1 At1b
O09175	AMPB_RAT	Aminopeptidase B (Arginine aminopeptidase)(Cytosol aminopeptidase IV)	Rnpep
P50123	AMPE_RAT	Glutamyl aminopeptidase (Aminopeptidase A) (AP-A) (CD antigen CD249)	Enpep
P15684	AMPN_RAT	Aminopeptidase N (AP-N) (Alanyl aminopeptidase) (Aminopeptidase M) (AP-M) (Kidney Zn peptidase) (KZP) (Microsomal aminopeptidase)	Anpep
P01015	ANGT_RAT	Angiotensinogen (Serpin A8) [Cleaved into: Angiotensin-1 (Angiotensin 1-10) (Angiotensin I) (Ang I); Angiotensin-2 (Angiotensin 1-8) (Angiotensin II) (Ang II); Angiotensin-3 (Angiotensin 2-8) (Angiotensin III) (Ang III) (Des-Asp[1]-angiotensin II); Angiotensin-4 (Angiotensin 3-8) (Angiotensin IV) (Ang IV); Angiotensin 1-9; Angiotensin 1-7; Angiotensin 1-5; Angiotensin 1-4]	Agt Serpina8
P29066	ARRB1_RAT	Beta-arrestin-1 (Arrestin beta-1)	Arrb1
P29067	ARRB2_RAT	Beta-arrestin-2 (Arrestin beta-2)	Arrb2
Q642A2	ATRAP_RAT	Type-1 angiotensin II receptor-associated protein (AT1 receptor-associated protein)	Agtrap Atrap
P25023	BKRB2_RAT	B2 bradykinin receptor (B2R) (BK-2 receptor)	Bdkrb2
P30099	C11B2_RAT	Cytochrome P450 11B2, mitochondrial (Aldosterone synthase) (CYPXIB2) (Cytochrome P450-Aldo-1	Cyp11b2 Cyp11b-2
P30100	C11B3_RAT	Cytochrome P450 11B3, mitochondrial (Aldosterone synthase) (CYPXIB3) (Cytochrome P450-Aldo-2	Cyp11b3 Cyp11b-3
P00787	CATB_RAT	Cathepsin B (EC 3.4.22.1) (Cathepsin B1 [Cleaved into: Cathepsin B light chain; Cathepsin B heavy chain]	Ctsb
P16228	CATE_RAT	Cathepsin E (EC 3.4.23.34)	Ctse

Entry	Entry name	Protein names	Gene names
P00786	CATH_RAT	Pro-cathepsin H [Cleaved into: Cathepsin H mini chain; Cathepsin H Cathepsin H heavy chain; Cathepsin H light chain]	Ctsh
P21961	CBPA3_RAT	Mast cell carboxypeptidase A (EC 3.4.17.1) (Carboxypeptidase A3)	Cpa3
P28234	CXA5_RAT	Gap junction alpha-5 protein (Connexin-40) (Cx40)	Gja5 Cxn-40
Q62737	CY24A_RAT	Cytochrome b-245 light chain (Cytochrome b(558) alpha chain) (Cytochrome b558 subunit alpha) (Neutrophil cytochrome b 22 kDa polypeptide) (Superoxide-generating NADPH oxidase light chain subunit)	Cyba
P19020	DRD3_RAT	D(3) dopamine receptor (Dopamine D3 receptor)	Drd3
P42893	ECE1_RAT	Endothelin-converting enzyme 1	Ece1
Q99372	ELN_RAT	Elastin (Tropoelastin)	Eln
P05370	G6PD_RAT	Glucose-6-phosphate 1-dehydrogenase (G6PD)	G6pdx G6pd
P54311	GBB1_RAT	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (Transducin beta chain 1)	Gnb1
P54313	GBB2_RAT	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (G protein subunit beta-2) (Transducin beta chain 2)	Gnb2
O35353	GBB4_RAT	Guanine nucleotide-binding protein subunit beta-4 (Transducin beta chain 4)	Gnb4
Q62689	JAK2_RAT	Tyrosine-protein kinase JAK2 (EC 2.7.10.2) (Janus kinase 2) (JAK-2)	Jak2
P00759	KLK2_RAT	Tonin (EC 3.4.21.35) (Esterase 1) (Glandular kallikrein-2)	Klk2 Klk-2 Ton
P97629	LCAP_RAT	Leucyl-cystinyl aminopeptidase (Cystinyl aminopeptidase) (EC 3.4.11.3) (GP160) (Insulin-regulated membrane aminopeptidase) (Insulin-responsive aminopeptidase)	Lnpep Irap Otase
Q9R0Q2	LT4R1_RAT	Leukotriene B4 receptor 1 (LTB4-R 1)	Ltb4r Blt
P12526	MAS_RAT	Proto-oncogene Mas	Mas1 Mas Mas-1
P09650	MCPT1_RAT	Mast cell protease 1 (rMCP-1) (EC 3.4.21.39) (Chymase))	Mcpt1
P97592	MCPT4_RAT	Mast cell protease 4 (rMCP-4) (EC 3.4.21.-) (Mast cell protease IV)	Mcpt4
P30904	MIF_RAT	Macrophage migration inhibitory factor (MIF) (EC 5.3.2.1) (Glutathione-binding 13 kDa protein) (L-dopachrome isomerase)	Mif
P07861	NEP_RAT	Neprilysin (EC 3.4.24.11) (Atriopeptidase) (Enkephalinase) (Neutral endopeptidase 24.11) (NEP	Mme
P42676	NEUL_RAT	Neurolysin, mitochondrial (Microsomal endopeptidase) (MEP) (Mitochondrial oligopeptidase M)	Nln
P14600	NK1R_RAT	Substance-P receptor (SPR) (NK-1 receptor) (NK-1R) (Tachykinin receptor 1)	Tacr1 Tac1r
P23441	NKX21_RAT	Homeobox protein Nkx-2.1 (Thyroid nuclear factor 1) (Thyroid transcription factor 1) (TTF-1)	Nkx2-1 Nkx-2.1 Titf1 Ttf-1 Ttf1

Entry	Entry name	Protein names	Gene names
P26824	PAR1_RAT	Proteinase-activated receptor 1 (PAR-1) (Thrombin receptor)	F2r Par1
Q63645	PAR2_RAT	Proteinase-activated receptor 2 (PAR-2) (Coagulation factor II receptor-like 1) (Thrombin receptor-like 1)	F2rl1 Par2
P41413	PCSK5_RAT	Proprotein convertase subtilisin/kexin type 5 (Proprotein convertase 5) (PC5) (Proprotein convertase 6) (PC6))	Pcsk5
O70196	PPCE_RAT	Prolyl endopeptidase (PE) (EC 3.4.21.26) (Post-proline cleaving enzyme) (rPop)	Prep
P08424	RENI_RAT	Renin (Angiotensinogenase)	Ren1 Ren
Q6AXS4	RENR_RAT	Renin receptor (ATPase H(+)-transporting lysosomal accessory protein 2) (ATPase H(+)-transporting lysosomal-interacting protein 2) (Renin/prorenin receptor)	Atp6ap2 Atp6ip2
F1M7S4	F1M7S4_RAT	Carboxypeptidase A3 (Mast cell carboxypeptidase A)	Cpa3 rCG_41643
Q68FT8	Q68FT8_RAT	Protein Serpinf2 (RCG33981, isoform CRA_a) (Serine (Or cysteine) peptidase inhibitor, clade F, member 2)	Serpinf2 rCG_33981
D3ZD62	D3ZD62_RAT	Protein Ndst2 (RCG41904, isoform CRA_c)	Ndst2 rCG_41904
B1WBL8	B1WBL8_RAT	Angiotensin II receptor, type 2 (Angiotensin II receptor, type 2, isoform CRA_a)	Agtr2 rCG_53260
D4A7G6	D4A7G6_RAT	Protein Gpr25 (RCG45981)	Gpr25 rCG_45981
C7ECU5	C7ECU5_RAT	Angiotensin I converting enzyme 2	Ace2 rCG_49658
Q91XT0	Q91XT0_RAT	E1A-binding protein (Protein Ep300)	Ep300 p300
D3ZYK4	D3ZYK4_RAT	Angiotensin-converting enzyme 2 (Fragment)	Ace2
Q9QX17	Q9QX17_RAT	Aminopeptidase N (Fragment)	Anpep APN
D3ZPX6	D3ZPX6_RAT	Elastin	Eln
D4AA31	D4AA31_RAT	Protein Prcp	Prcp
F1LPS4	F1LPS4_RAT	Cytochrome P450 11B2, mitochondrial	Cyp11b2
Q9R258	Q9R258_RAT	Renin b (Fragment)	Ren Ren1
Q6BDA5	Q6BDA5_RAT	Dual endothelin 1, angiotensin II receptor	Dear
D4A9U4	D4A9U4_RAT	Elastin	Eln
Q9JIE2	Q9JIE2_RAT	Renin (Fragment)	Ren Ren1
Q9QX18	Q9QX18_RAT	Aminopeptidase N (Fragment)	Anpep APN

Table 19. RAS related gene ontology terms. The associated protein names were found using the UniProt database.

APPENDIX 3:

CONFIRMATION OF CHEMICAL REACTIVITY OF ANGII STOCK SOLUTION

Measuring AngII induced cytosolic Ca^{2+} flux

In order to verify the chemical reactivity of the AngII stock solution used for respirometry experiments, dynamic measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were made using the cell permeant green-fluorescent Ca^{2+} indicator Fluo-4 AM.

HepG2 cells were cultured in RPMI 1640 with Glutamax (Life Technologies, Carlbad, Ca, USA) supplemented with 5% heat inactivated fetal calf serum (FCS, Biological Industries, Beir Haemak, Isreal) and antibiotics (Penicillin-Streptomycin (50 U/mL)). For $[Ca^{2+}]_I$ measurements, cells were loaded with 4 µM Fluo-4 AM for 30 minutes at 37 ˚C in Krebs-Ringer modified buffer containing 1 mM $CaCl_2$ (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM K_2HPO_4, 1 mM $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES [pH 7.4]).

Loaded cells were imaged with an Olympus IX71 microscope with a 40x lens, and images were captured using a Sensicam QE cooled digital CCD camera (Cooke, Romulus, MI) at 2.6 seconds intervals. An excitation wavelength of 488 nM was used and emitted fluorescence collected above 505 nm. Cells were maintained at 37 ˚C.

The Fluo-4 signal was measured before and after addition of 1 µM AngII (# 151039 M005, Alexis biochemicals, Exeter, UK). The mitochondrial ETS uncoupler *p*-Trifluoromethoxy-phenylhydrazone (FCCP), which releases mitochondrial Ca^{2+} into the cytosol was used as a control condition.

Images were analysed using Metamorph data acquisition software (Visitron Systems, West Chester, PA, USA). Ten regions of interest (ROI) were selected (10 cells) and the Fluo-4 AM fluorescence intensity was measured.

As shown in Fig 34, 1 µM AngII caused a peak in $[Ca^{2+}]_I$ confirming that the chemical was stable and reactive at 37 C.

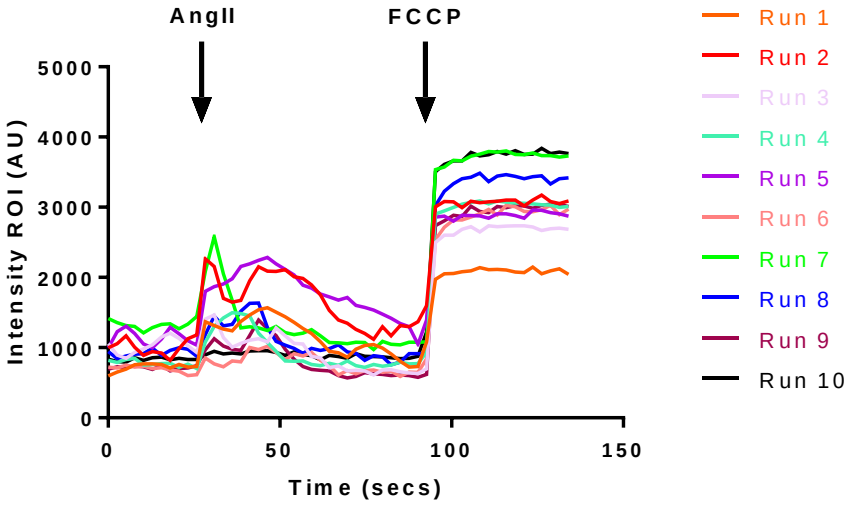

Figure 33. Cytosolic Ca^{2+} $[Ca^{2+}]_i$ measurements in HepG2 cells stimulated by 1 µM AngII. HepG2 cells were loaded with 4 µM Fluo-4 for 30 minutes. Cells were excited at 488 nM wavelength light and imaged using a confocal microscope. Images were acquired during stimulation with both 1 µM AngII and 1 µM FCCP. Regions of interest (ROI) were selected and signal intensity measured. The increase in intensity following AngII addition indicates $[Ca^{2+}]_I$ release and therefore chemically reactive AngII.

APPENDIX 4:

PUBLISHED PAPERS ARISING FROM THIS WORK

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENERGY METABOLISM
HORMONE RECEPTORS
MEDICAL RESEARCH
BIOCHEMISTRY

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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 pressure[1]. 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 novo[2–5]. Whether of local or systemic origin, AngII mediates its effects through action at two receptor subtypes. While the role of its type-2 receptor (AT_2R) is less clear, the type-1 receptor (AT_1R) 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 environments[8–12]. In rodents, combined ACE inhibition and AT_1R antagonism reduce renal oxygen consumption related to sodium transport[13], 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 AT_1Rs have been observed on the outer mitochondrial membrane (OMM)[20,21], and that exogenously-administered ^{3}H-labelled AngII has been shown to traffic to the surface of rodent mitochondria[22]. 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 AT_2Rs on the inner mitochondrial membrane[23]. However, this conclusion was largely based upon the use of AT_2R 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 AT_1R 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 livers[24]. 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 species[27], 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 proteins[28]. 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.

AT_1R is present in the MAM, but no AT_1R, AT_2R 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 [^{125}I]-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 membrane[29], 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 AT_1R antagonist Losartan, but not affected by the AT_2R antagonist PD123319, indicating that binding was AT_1R-related (Fig. 2B).

In order to further analyse the presence and localization of AT_1R and AT_2R 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 AT_1R in the CM and MAM fraction, but not in the PM (Fig. 2C). In contrast, antibodies against AT_2R 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-AT_2R antibody pulled down three bands in the range of 30–70 kD in the MAM and partly in the PM fraction

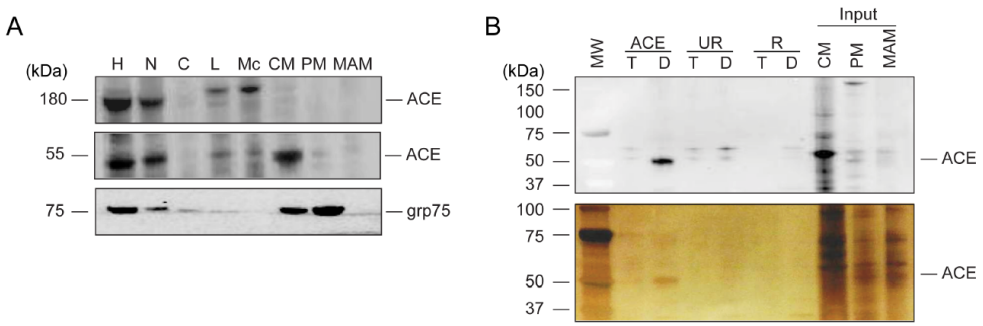

Figure 1 | Mitochondrial subfractions lack detectable ACE. (A). Western blot using anti ACE and anti-grp75 antibodies from rat liver subcellular fractions. H-homogenate, N-nuclear fraction, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation to obtain 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 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 (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.

A

Pooled data (n=3)	K_D (nM)	B_{MAX} (fmol/mg)	nH
PM	0.38 ± 0.30	0.42 ± 0.06	0.90 ± 0.17
MAM	0.28 ± 0.12	15.34 ± 5.1	1.01 ± 0.03

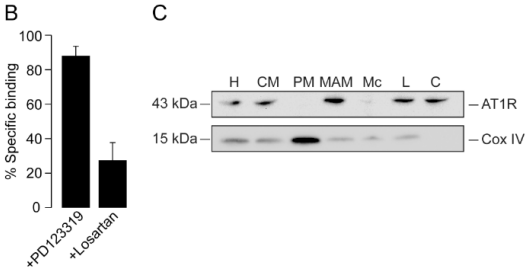

Figure 2 | AT_1R is present in the MAM fraction. (A). Affinity (K_D), density (B_{MAX}) and cooperativity (Hill coefficient, nH) of specific AngII binding sites in the PM and MAM fractions. (B). Specific binding of [^{125}I]-AngII in the MAM fraction in the presence of AT_1R (losartan) or AT_2R (PD123319) blockers. The concentration of losartan was calculated from inhibition constants[29] to block >95% of the AT1 receptor but <2% of AT_2R. [PD123319] was calculated to block 99% of AT_2R but <2% of AT_1R. (C). Western blot detection of AT_1R 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 AT_1Rs 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 µM) 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 µM 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 µM 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.

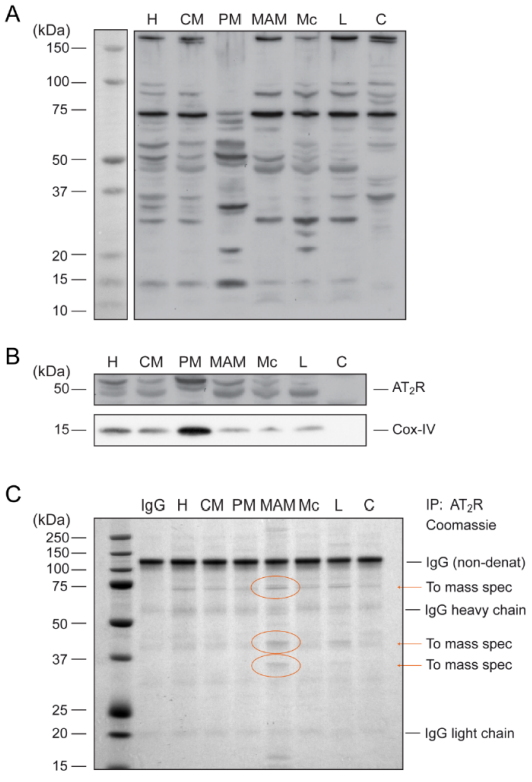

Figure 3 | Lack of an AT_2R specific immunoreactive band in rat liver mitochondria. (A). Immunoreactive bands detected by the sc9040, rabbit AT_2R antibody in subcellular fractions: H-homogenate, C-cytosol, L-lysosomes, Mc-microsomes. Rat liver mitochondria were purified by differential centrifugation (CM) and further separated to mitochondria associated membranes (MAM) and pure mitochondrial (PM) fractions by isopicnic 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 AT_2R. The AT_2R 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). AT_2R 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-denaturating 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 mitochondria[32]. Moreover, intra-mitochondrial 'dense bodies' were shown to contain renin using immunogold labelling and electron microscopy (EM)[33], together with angiotensinogen and AngII[34]. 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 secretion[36]. 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 fraction[37], 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 endosymbiontic 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 predicted[39], 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 & Cook[18]), 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 1970's[22], and later confirmed by several groups in a range of tissues, along with AT_1Rs[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 AT_1R receptors is linked to intracellular Ca^{2+} mobilisation following Ca^{2+} release from endoplasmic reticulum Ca^{2+} stores[19]. The ensuing accumulation of Ca^{2+} in mitochondria can exert acute activation of the Krebs cycle and OXPHOS[41], but chronic activation of the pathway may lead to mitochondrial Ca^{2+} overload, depolarisation and reduced ATP production[42,43]. Parallel to Ca^{2+} signalling, multiple pathways are activated by both AT_1R and AT_2R, 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

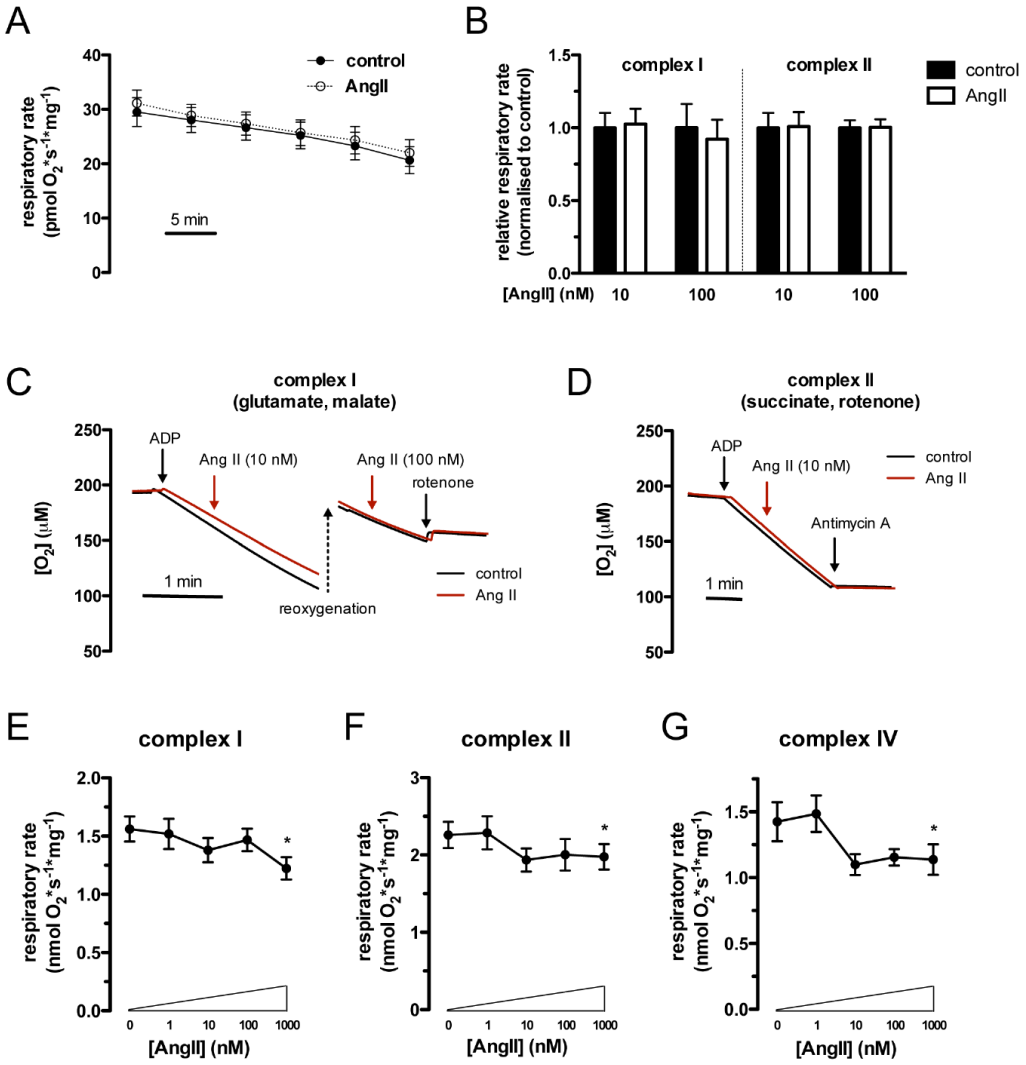

Figure 4 | The effect of AngII on respiration of isolated liver mitochondria. (A). Basal oxygen consumption of isolated rat liver mitochondrial 5 min after transfer to the respiration chamber, and at 5, 10, 15, 20 and 25 minutes after addition of AngII (1 µM) (n = 18). (B–D). ADP-induced oxygen consumption 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 µM)). Data represent mean ±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, AT_1Rs 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 AT_2Rs on mitochondria from rat heart and liver[23]. 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 AT_2R agonist, which has been reported to antagonise the AT_1R as well[45], 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). [^{125}I]Tyr^4-Angiotensin II (2200 Ci/mmol, 81.4 TBq/mmol) was from PerkinElmer (Cambride, UK). Primary antibodies were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany; AT_1R: sc1173, rabbit; AT_2R: 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 centrifugation[46]. 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^{TM} 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 described[24]. 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 ul 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 AT_1R and AT_2R, samples were solubilized using ice-cold buffer (50 mM HEPES, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 30 mM $PyroPO_4$, 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-denaturating 4× loading buffer without dithiothreitol and β-mercaptoethanol (200 nM 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 described[47]. 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 as complete a 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 $MgCl_2$, 80 mM KCl, 60 mM K-lactobionate, 10 mM KH_2PO_4, 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*-phenylendiamine (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 (NaH_2PO_4, 50 mM; NaCl, 100 mM; EGTA, 1 mM; $MgCl_2$, 10 mM; BSA, 0.2%; pH 7.4) with increasing concentrations (15 pM-2 nM) of [^{125}I]-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 [^{125}I]-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 AT_1 vs. AT_2 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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Additional information

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