

Identification and characterization of a functional mitochondrial angiotensin system

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The renin-angiotensin (Ang) system regulates multiple physiological functions through Ang II type 1 and type 2 receptors. Prior studies suggest an intracellular pool of Ang II that may be released in an autocrine manner upon stretch to activate surface membrane Ang receptors. Alternatively, an intracellular renin-Ang system has been proposed, with a primary focus on nuclear Ang receptors. A mitochondrial Ang system has not been previously described. Here we report that functional Ang II type 2 receptors are present on mitochondrial inner membranes and are colocalized with endogenous Ang. We demonstrate that activation of the mitochondrial Ang system is coupled to mitochondrial nitric oxide production and can modulate respiration. In addition, we present evidence of age-related changes in mitochondrial Ang receptor expression, i.e., increased mitochondrial Ang II type 1 receptor and decreased type 2 receptor density that is reversed by chronic treatment with the Ang II type 1 receptor blocker losartan. The presence of a functional Ang system in human mitochondria provides a foundation for understanding the interaction between mitochondria and chronic disease states and reveals potential therapeutic targets for optimizing mitochondrial function and decreasing chronic disease burden with aging.

frailty | ATP | bioenergetics

The renin-angiotensin (Ang) system (RAS) is a key regulator of cardiovascular and renal function. Although many studies have focused on the impact of extracellular Ang II and its receptors, type 1 (AT₁R) and type 2 (AT₂R), on the cardiovascular system, others have reported that Ang II is also present in the intracellular compartment and can be released upon cell stretch to mediate cellular growth and/or apoptosis (1–3). Although many of the autocrine effects of this endogenous Ang store are believed to be mediated by plasma membrane Ang receptors, an intracellular RAS acting on nuclear Ang receptors has also been proposed (4, 5).

The RAS influences cardiovascular function via nitric oxide (NO) regulation (6–8). AT₁R blockade increases NO, and this increase is abolished by concomitant AT₂R blockade, suggesting that AT₂Rs are important in NO production (9). AT₂Rs likely increase NO production via direct stimulation of NO synthase (NOS) (10) or indirectly through bradykinin-dependent mechanisms (7). Recently, the intracrine activation of AT₂Rs has been reported to increase NO production in isolated cortical kidney nuclei (11).

Possible sources of NO coupled to Ang signaling include the three canonical NOS isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) NOS (12). Additionally, there have been reports of a NOS isoform in mitochondria (mtNOS) (13, 14). Although the unique identity of mtNOS is still controversial (15), this mitochondria-specific isoform is localized to the inner mitochondrial membrane, where it may regulate mitochondrial respiration (13, 16, 17). mtNOS activity is thought to decline with aging (18). Although several studies have provided evidence that Ang receptors can couple to the canonical NOS isoforms (7), nothing is known about whether intracellular Ang II influences mitochondrial NO production or if it has any other

effects on mitochondrial function. Given the known relationship between the RAS and cellular NO production, as well as the emerging understanding of intracellular RAS functionality, we hypothesized that the RAS plays an important role in intramitochondrial NO production.

Results

Several approaches were used to test our hypothesis on the presence and function of a mitochondrial Ang system (MAS). First, using high-resolution transmission immunoelectron microscopy and well-characterized antibodies against AT₁R, AT₂R, and Ang, we studied sections from human skeletal muscle cells and monocytes as well as mouse cardiac myocytes, renal tubular cells, neuronal cells, vascular endothelial cells, and hepatocytes. Second, using double-labeling and immunoelectron microscopy, we demonstrated colocalization of Ang receptor(s) and Ang. Third, we transfected human fibroblasts with a pcDNA-Cycle 3 GFP-AT₂R construct, leading to expression of Cycle 3 GFP-AT₂R, which we tracked by using confocal imaging in living cells. Fourth, we purified mitochondrial subfractions down to the inner mitochondrial membrane to determine the precise location of the Ang receptors within the mitochondria. Fifth, we measured changes in mitochondrial NO production and respiration in response to activation or inhibition of the receptor(s) in isolated mitochondria. Finally, we characterized the effects of aging and long-term blockade of AT₁Rs on the MAS.

Subcellular Distribution of AT₁Rs and AT₂Rs. The subcellular distribution of AT₁Rs and AT₂Rs was assessed by high resolution immunoelectron microscopy in human monocytes, skeletal myocytes and in cardiac myocytes, renal tubular cells, neuronal cells, vascular endothelial cells, and hepatocytes from C57BL/6 mouse, using specific polyclonal antipeptide antibodies. AT₁R immunoreactivity was observed in the cell membrane of human monocytes (Fig. 1A). AT₁R did not appear in the mitochondria from young adult monocytes or animal tubular kidney cells (Fig. 1B and C) except in rare occurrences (Fig. 1D), but was consistently found not far from the mitochondrial outer membrane (Fig. 1C). AT₂R immunoreactivity was observed in the cell membrane of human monocytes (Fig. 2A). Abundant mitochondrial AT₂R (mtAT₂R) was also observed in human monocytes and mouse tissues (Fig. 2B–D). To confirm the immunoelectron microscopy findings, mouse heart homogenates were fractionated first by differential centrifugation and sub-

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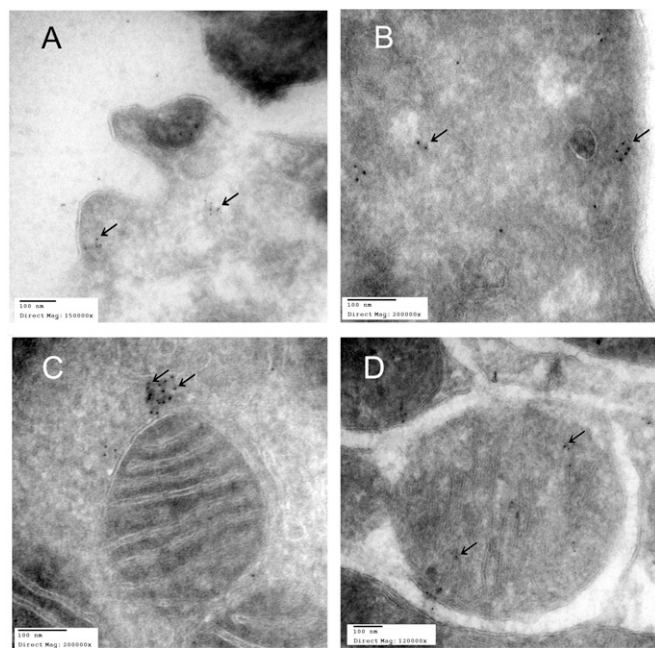


Fig. 1. Immunoelectron microscopic localization of AT₁Rs in sections of human monocyte (A and B) and mouse kidney tubular cells (C and D) using gold bead labeling (arrows) for AT₁Rs. A shows AT₁Rs on human monocyte cell membrane and in the cytoplasm (B). C shows labeling in close proximity to mitochondria and rarely in the mitochondria (D).

sequently by density gradient centrifugation. The density-purified mitochondria were probed with specific antibodies. AT₂R immunoreactivity increased with progressive purification of mitochondria, for which cytochrome *c* oxidase (CoxIV) was used as a marker. Importantly, the immunoreactivity of Na⁺/K⁺ ATPase, a plasma membrane marker, declined with progressive enrichment of mitochondria. This is consistent with mitochondrial enrichment of AT₂Rs (Fig. S1A and B). In contrast, AT₁Rs did not enrich with mitochondrial fractions.

We quantified through EM the relative expression of AT₂Rs per mitochondria in cells obtained from different human and mouse tissues (Fig. S1C).

Colocalization of Ang and AT₂Rs. Given that a functional MAS would require the presence of local Ang to activate the receptor, we next sought evidence for mitochondrial Ang by immunoelectron microscopy. Using a gold-conjugated secondary antibody to anti-Ang antibody (6 nm gold), we demonstrated the presence of mitochondrial Ang in mouse hepatocytes (Fig. 3A), kidney tubular cells (Fig. 3B), brain neurons (Fig. 3C), and heart myocytes (Fig. 3D). Moreover, Ang was not randomly distributed within mitochondria; rather, it colocalized with mtAT₂R, detected with a gold-conjugated secondary antibody to anti-AT₂R antibody (12 nm gold). The abundant distribution of mtAT₂R in the young animals, observed in electron microscope images, was correlated with a scarcity of mtAT₁R. Despite the observation of many Ang immunolabeled particles in electron microscope images, Ang was not detected in Western blots of isolated, density-purified mitochondria, indicating that Ang is loosely bound and is lost upon washing the isolated mitochondria.

AT₂R Transfection of Fibroblast Cells. To ensure that the results described above were not attributable to nonspecific or off-target binding of antibody to mitochondrial targets, we confirmed mitochondrial localization of AT₂Rs by using a GFP-AT₂R fusion construct. Briefly, human fibroblasts were transfected with pcDNA-Cycle 3 GFP-AT₂R or positive control using pcDNA-EGFP-C1 before counterstaining with the mitochondrial fluo-

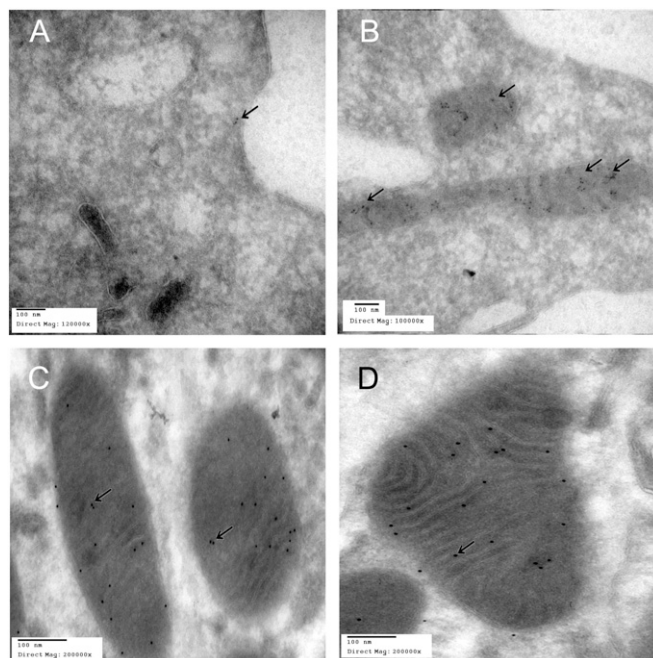


Fig. 2. Immunoelectron microscopic localization of AT₂Rs in sections of human monocyte (A and B) and mouse renal tubular cells (C and D) using gold beads labeling (arrows) for AT₂Rs. A shows AT₂Rs on human monocyte cell membrane, and B–D reveal heavy labeling for AT₂Rs within mitochondria.

rescence marker MitoTracker Red. GFP-AT₂R fluorescence was confined to discrete intracellular puncta (Fig. 4B), mirroring the distribution of MitoTracker Red (Fig. 4A). Spatial overlap of the two signals, denoting subcellular colocalization, is shown in Fig. 4C. The extent of colocalization was assessed quantitatively over a series of confocal Z sections (0.37 μm); spatial signal correlation between the two fluorophores was high ($R^2 = 0.72$). Thus, the high density of AT₂Rs observed in the mitochondria in electron microscope images was corroborated by tracking the Cycle 3 GFP-AT₂R fusion protein in live human fibroblasts, where it was predominantly colocalized with the mitochondrial marker MitoTracker Red.

AT₂R Localization Within Mitochondria. Having confirmed the presence of mtAT₂Rs by two independent methods, we sought to determine the precise location of these receptors within the mitochondria. Further inspection of the immunoelectron micrographs in Figs. 2 and 3 suggested that the mtAT₂R is resident in the inner, rather than the outer, mitochondrial membranes. To confirm this observation by a different method, we isolated inner mitochondrial membrane-enriched membranes from mouse liver according to previously published methods (19). We determined the specific immunoreactivity of AT₂R in the inner mitochondrial membrane as described in the previous section. AT₂R immunoreactivity copurified with that of ATP synthase β, an inner mitochondrial membrane marker. In contrast, markers of the plasma membrane (Na⁺/K⁺ ATPase) and the outer mitochondrial membrane [voltage-dependent anion channel (VDAC)] were progressively removed by inner mitochondrial membrane purification (Fig. 5A). These results buttress the results of the immunoelectron microscopy studies and are consistent with colocalization of AT₂R in the inner mitochondrial membrane.

AT₂Rs Modulate Mitochondrial NO Production. To determine whether mtAT₂R activation might modulate mitochondrial NO production in a manner analogous to the actions of non-mtAT₂Rs on other NO isoforms, we examined the direct effects of AT₂R agonists and antagonists on isolated kidney mitochondria. Trans-

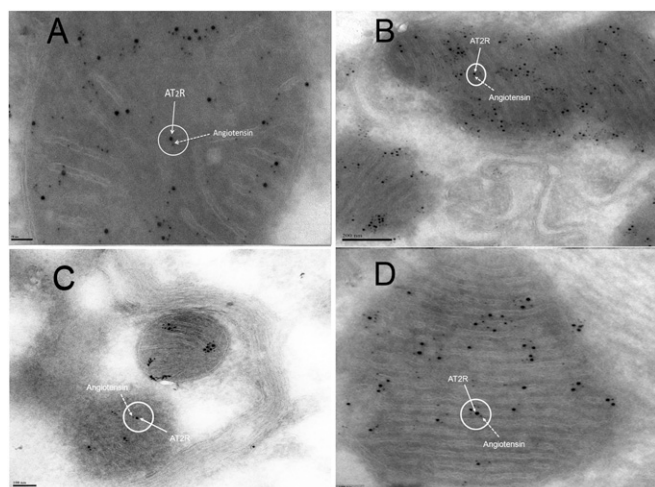


Fig. 3. Immunoelectron microscopic localization of AT₂R binding to Ang in the mitochondria by using a gold-labeled anti-AT₂R antibody (12 nm gold) and a gold-labeled anti-Ang antibody (6 nm gold). Shown is colocalization of AT₂R with Ang in sections of mouse hepatocytes (A), kidney tubular cells (B), neurons (C), and cardiac myocytes (D).

mission electron microscopy and Western blot analyses were used to confirm minimal contamination of the isolated mitochondria with other cell fractions and a typical morphology of mitochondria. The NO fluorescent molecular detection probe kit (Enzo Life Sciences) was used according to the manufacturer's instructions (20). Isolated mitochondria were treated for 30 min with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (c-PTIO) followed by a 15-min incubation with the AT₂R agonist CGP421140 and/or the antagonist PD-123319. Positive control samples were treated with the NOS substrate L-arginine, and negative control samples were generated by treatment with NO scavenger (c-PTIO). Isolated mitochondria were treated and then incubated with a specific fluorescent probe for real-time measurement of NO. Fluorescence signal was measured by using a multimode microplate reader equipped with Cyanine 5 (650/670 nm). Treatment of mitochondria with CGP421140 caused a concentration-dependent increase in mitochondrial NO production (Fig. 6A); specifically, 10 nM was sufficient to stimulate NO production [from 0.52 ± 0.0009 fluorescence arbitrary units (AU) at baseline control to 0.6 ± 0.01 AU, $P < 0.001$]. Moreover, CGP421140-stimulated NO production was mitigated by pre-treatment with PD-123319 (1 μ M; 0.55 ± 0.006 AU, $P < 0.01$; Fig. 6A).

AT₂Rs Modulate Mitochondrial Respiration. To determine how mtAT₂R stimulation might influence global mitochondrial function, we examined effects on respiration in rat heart mitochondria. Glutamate/malate-supported respiration (state 2) (21) was initially 17.8 ± 1.06 pmol/min per μ g. Addition of ADP increased respiration (state 3) to 84.12 ± 1.24 pmol/min per μ g ($P < 0.0005$). We subsequently found that the addition of 100 nM CGP421140 caused a significant decrease in state 3 respiration (Fig. 6C; 39.47 ± 4.37 pmol/min per μ g, $P < 0.005$). The dose of 100 nM CGP421140 was selected based on a series of experiments used to determine the dose of CGP421140 needed to influence mitochondrial respiration (Fig. 6B). The effects of CGP421140 were not likely attributable to nonspecific uncoupling because control experiments showed that 100 nM CGP421140 had little effect on mitochondrial membrane potential ($\Delta\Psi_m$) or NADH level (Fig. 6D). Importantly, CGP421140-mediated inhibition of respiration was prevented by blocking the AT₂R; PD-123319 (1 μ M) restored CGP421140-inhibited state 3 respiration (Fig. 6C; 66.83 ± 2.1 pmol/min per μ g, $P < 0.01$). The addition of L-N^G-nitroarginine methyl ester (L-NAME; 1 μ M), an arginine analog that inhibits NO production, reversed the CGP421140 effect on mitochondrial respiration (Fig. 6C; 67.4 ± 4.3 pmol/min per μ g, $P < 0.05$), indicating that the functional effects of mtAT₂R activation on mitochondrial respiration are via an NO-dependent mechanism.

To further validate this effect of MAS on mitochondrial respiration, we studied liver mitochondria with different mitochondrial substrates. Liver mitochondria respiring with glutamate/malate as the substrate gave results similar to those obtained in isolated cardiac mitochondria. The AT₂R agonist inhibited state 3 respirations in the nanomolar range for both glutamate/malate- and succinate-supported respiration. In succinate-supported respiration, the inhibitory response was slightly more pronounced at 1 nM than for glutamate/malate-supported respiration (Fig. S2).

Age-Related Changes in the MAS. Although still controversial, substantial evidence documents a decline in the canonical Ang system with age (22–24), and mtNOS activity is reportedly reduced with aging (18). We examined the effect of aging on the MAS. Kidney tubular cell sections from 20- or 70-wk-old C57BL/6 mice and aged (70-wk-old) mice treated with losartan at a dose of 40–60 mg/kg per day for 20 wk were labeled with antibodies and visualized with immunoelectron microscopy. Representative mitochondria, labeled for AT₁R (Fig. 7A–C) and AT₂R (Fig. 7D–F) are shown. Younger mice (Fig. 7A and D) and older mice treated with losartan (Fig. 7C and F) had similar densities of labeled AT₂R, whereas older untreated mice (Fig. 7B and E) had a lower AT₂R density. Our results demonstrate a significant decrease in the expression of mtAT₂R with aging (from 25.5 +

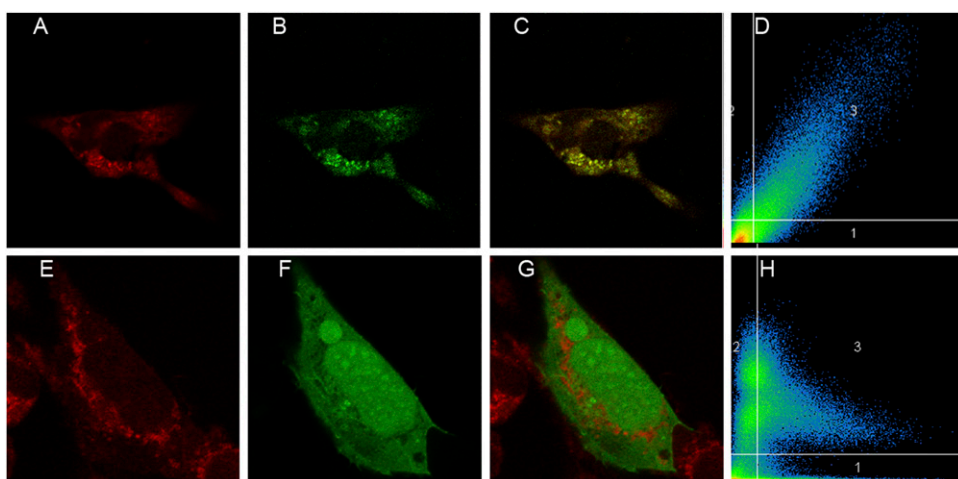


Fig. 4. Transfected AT₂Rs colocalize with mitochondria in human fibroblasts. Human fibroblast cells were transfected with pcDNA-Cycle 3 GFP-AT₂R construct (B) or positive control using pcDNA-EGFP-C1 (F) and counterstained with MitoTracker Red (A and E) (100 \times oil immersion). The merged images show yellow fluorescence (C and G). Fluorographic analysis (D and H) reveals a high correlation coefficient ($R^2 = 0.72$), suggesting a strong colocalization between AT₂Rs and MitoTracker within the mitochondria.

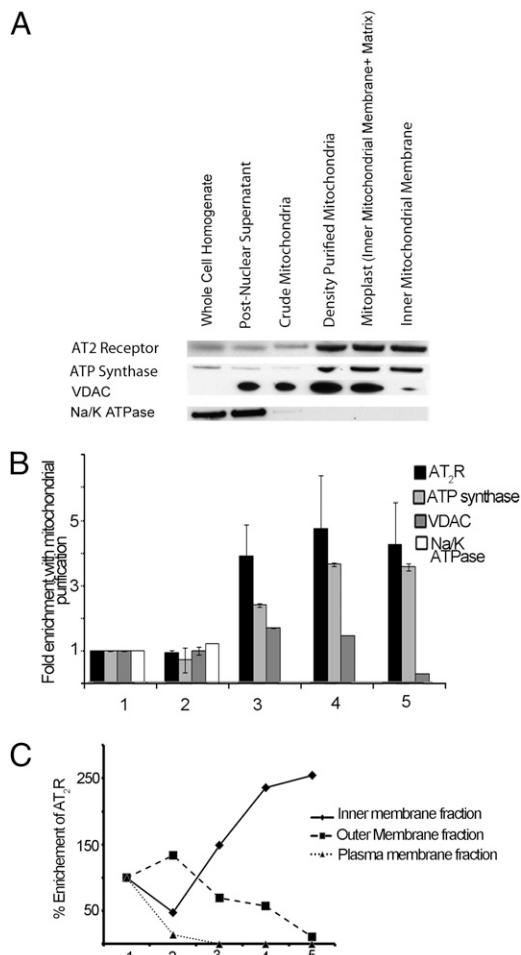


Fig. 5. Purification of inner mitochondrial membrane AT₂Rs. (A) Whole-liver homogenate fractionations up to the inner mitochondrial membrane were subjected to 12% SDS/PAGE and immunoblotting with anti-AT₂R as well as anti-Na⁺/K⁺ ATPase, anti-VDAC, and anti-ATP synthase β for detecting cell membrane, outer mitochondrial membrane, and inner mitochondrial membrane markers, respectively. AT₂Rs tracked with inner mitochondrial membrane marker ATP synthase β , consistent with inner mitochondrial membrane localization of AT₂Rs. (B) Integrated densitometric band analysis of immunoblots demonstrating fold enrichment of AT₂Rs, inner mitochondrial membrane marker (ATP synthase), outer mitochondrial membrane marker (VDAC), and plasma membrane marker (Na/K ATPase) with mitochondrial purification. (C) Percentage enrichment of AT₂Rs with cellular subfractions through mitochondrial purification. Fractions: 1, whole-cell lysate; 2, postnuclear (480 \times g); 3, postdifferential centrifugation (7,700 \times g); 4, post-HistoDenz gradient centrifugation (50,500 \times g); 5, postsucrose gradient centrifugation (77,000 \times g).

6.2 to 10.2 + 2.2 gold-labeled AT₂R per mitochondrion, $P < 0.001$) that is reversed by treatment with losartan (39.0 + 2.6 gold-labeled AT₂R per mitochondrion). In contrast, mtAT₁R was significantly increased with aging (from 2.0 + 2.2 to 12.7 + 5.8 gold-labeled AT₁R per mitochondrion, $P < 0.001$). This increase was slightly attenuated with chronic AT₁R blockade (11.0 + 2.1 gold-labeled AT₁R per mitochondrion).

Discussion

Virtually every organ system in the human body has been shown to possess a local Ang signaling system. The components of the local Ang systems are present in peripheral tissues such as the vasculature, kidneys, adrenal glands, liver, heart, and immune cells, all of which locally produce Ang II. An intracellular RAS has also been proposed, whereby Ang II produced by intracellular angiotensinogen acts on nuclear Ang receptors (11, 25).

The present findings, which document the subcellular localization of functional MAS coupled to AT₂R, open an area of investigation into the regulation of mitochondrial function by Ang II-mediated intracrine signaling. Although previous reports do not specifically anticipate the localization of Ang receptors on mitochondria, some clues may be gleaned from prior work (5, 26–29). Early studies indicated that radio-labeled Ang II could be associated with the mitochondria (30), and chronic exposure to Ang II had a significant stimulatory effect on rat mitochondrial growth and proliferation (31) as well as improved coupling of respiration and ATP synthesis in intact mitochondria (27).

How these receptors are transported to the mitochondria is still unclear. Many mitochondrial proteins are synthesized as cytosolic precursors, containing N-terminal mitochondrial localization sequences. Cytosolic chaperones then deliver the precursor proteins to the transport machinery of the mitochondrial outer and inner membranes. Neither AT₁Rs nor AT₂Rs possess a consensus targeting sequence. Nevertheless, a number of other mitochondrial transmembrane proteins [e.g., mitochondrial carrier proteins such as adenine nucleotide translocase (32)] also do not contain a predictable mitochondrial signal sequence (33), and the absence of this criterion does not exclude trafficking to the mitochondria.

The localization of AT₂Rs in the mitochondrial inner membrane suggests their importance to NO production, which is believed to originate in the inner membrane through mtNOS (16, 34, 35). NO production was dose-dependently activated by the AT₂R agonist CGP421140 in isolated mitochondria, and this effect was antagonized by AT₂R blocker PD-123319. The observation that there was no change in $\Delta\Psi_m$ or NADH and that mitochondrial respiration remained well-coupled at much higher doses of CGP421140 makes it unlikely that the observed responses were attributable to nonspecific toxic effects of CGP421140 on mitochondrial energetics. Moreover, because the NO response occurred in isolated mitochondria and the AT₂R was localized to the inner mitochondrial membrane, the data could be taken as support for a mitochondrially localized NOS, a currently controversial subject. Whether AT₂R-coupled NO is produced on the matrix side of the inner membrane remains to be determined.

Mitochondrial NO production has been reported to reversibly inhibit mitochondrial respiration by binding to CoxIV (36). We observed inhibition of respiration by the AT₂R agonist in parallel with NO production. Importantly, CGP421140-reduced respiration was reversed by the addition of the NO blocker L-NAME, indicating that the functional effects of mtAT₂R activation on mitochondrial respiration is via an NO-dependent mechanism. Alternatively, downstream effects of NO, such as activation of cGMP production and posttranslational modification of mitochondrial targets, could also account for the observed decrease in oxygen consumption. Further investigation will be necessary to determine whether the AT₂R effect on respiration depends on NO or cGMP.

The decrease in mtAT₂R and increase in mtAT₁R density with aging, and the reversal of these changes with long-term AT₁R blockade, fits with the general model of antagonism between AT₁Rs and AT₂Rs (37) as well as with reports that AT₁R blockers up-regulate AT₂R expression and function (38). In addition, AT₁R blockers have been reported to reduce age-related mitochondrial dysfunction, attenuate hypertension-induced renal mitochondrial dysfunction, and protect against cardiac mitochondrial dysfunction in the setting of acute ischemia (39, 40). Interestingly, disruption of AT₁Rs was associated with an increased number of mitochondria and up-regulation of the prosurvival genes nicotinamide phosphoribosyltransferase (*Nampt*) and sirtuin 3 (*Sirt3*) in the kidney, leading to marked prolongation of life span in mice (41).

Although Ang II is the major effector peptide of the RAS, recent evidence suggests that its metabolite des-aspartyl¹-Ang II (Ang III) plays an important role in the activation of AT₂Rs (42). Ang III is more membrane-permeable than Ang II and the most prevalent Ang peptide in the brain, and perhaps inside the cell,

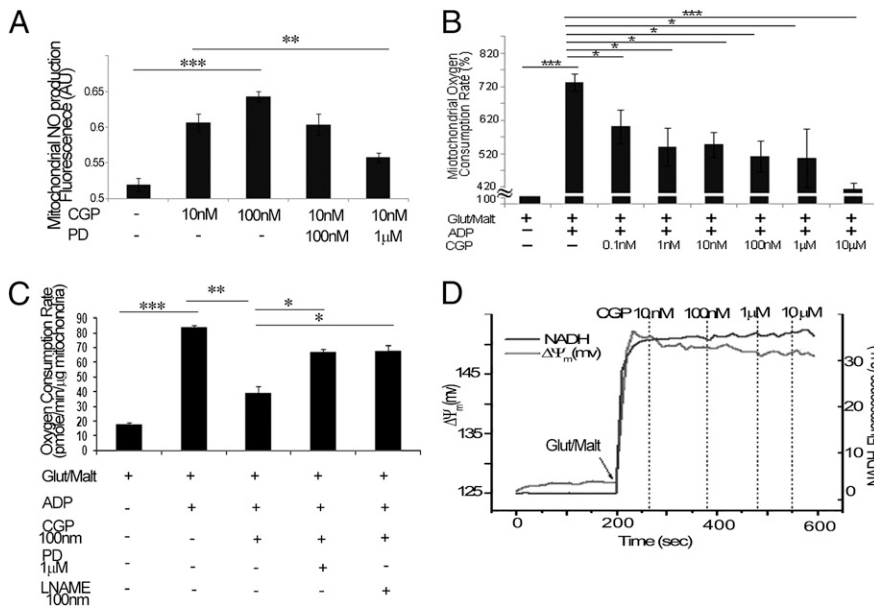


Fig. 6. mtAT₂R modulation of mitochondrial respiration and NO production. (A) Increased mitochondrial NO production in response to 10 nM and 100 nM concentrations of the AT₂R agonist CGP421140 (CGP), which can be reversed with the addition of a 1 μM concentration of the AT₂R antagonist PD-123319 (PD). (B) Mitochondrial respiration decreased significantly in response to serially increasing concentrations of CGP421140. Linear regression of CGP421140 concentrations versus oxygen consumption was significant at $P < 0.0004$. (C) Decreased respiration in response to AT₂R agonist CGP421140 at 100 nM was reversed with the addition of AT₂R antagonist PD-123319 at 1 μM or an inhibitor of NO production, L-NAME, at 100 nM. (D) No changes in mitochondrial membrane potential ($\Delta\Psi_m$) are evident in response to CGP421140 at increasing concentrations, confirming that effects observed on mitochondrial respiration are not attributable to nonspecific effects of CGP421140 on mitochondrial bioenergetic parameters. (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.)

because of its lipophilicity (43, 44). Because of the minimal structural difference between Ang II and Ang III, it is difficult to distinguish between the two peptides via most Ang II-specific antibodies. Given this fact and the evidence of the important role that Ang III plays in activation of the Ang system (44), the effects seen in the MAS may perhaps be driven by Ang III rather than Ang II. Hence, the role that the active metabolites of Ang II play in the activation of the MAS will require further study.

A recent study reported that AT₂R can act as either an antioxidative or a prooxidative receptor (45), depending on the conditions. Further studies are needed to determine the role of the MAS in the production of ROS and its potential effects in the mitochondria and cellular environment.

In the light of our present study and recent reports on the effects of AT₁R blockers on mitochondrial number and function, such effects might be mediated via unopposed mtAT₂Rs. Given the established role of NO in cardioprotection (46), it is tempting to speculate that the MAS could be involved in mitigation of ischemic or age-related mitochondrial injury. Although many more studies are required to define the role that this system plays in the function of mitochondria in each organ system, the identification and characterization of a functional MAS opens up avenues of research involving the Ang system.

Methods

The following sections contain a brief overview of the methods used for results presented in this article. For further details of these methodologies, please see *SI Methods*.

Isolation of Mitochondria. For functional assays, crude mitochondria from animal groups were separated by using differential centrifugation (47). For structural studies, mouse liver, heart, kidney, and brain cells were subjected to fractionation (19).

Western Blot Analysis. Mitochondrial preparations were resolved by 12% SDS/PAGE, transferred to the nitrocellulose membranes, and probed against Na⁺/K⁺ ATPase, CoxIV, VDAC, ATP synthase β , AT₂R, Ang, and AT₁R.

Transfection of Human Fibroblast Cells. Human fibroblast cells were transiently transfected with pcDNA-Cycle 3 GFP-AT₂R, positive control pcDNA-EGFP, or negative control pcDNA AT₂R-Cycle 3 GFP. Images were acquired with the Zeiss Meta Confocal Microscope System.

Mitochondrial NO Production. Isolated mitochondria were incubated with AT₂R agonists (CGP421140 at 10 nM and 100 nM) and antagonists (PD-123319 at 100 nM and 1 μM). The NO fluorescent molecular detection probe kit (Enzo Life Sciences) was used according to the manufacturer's instructions (20).

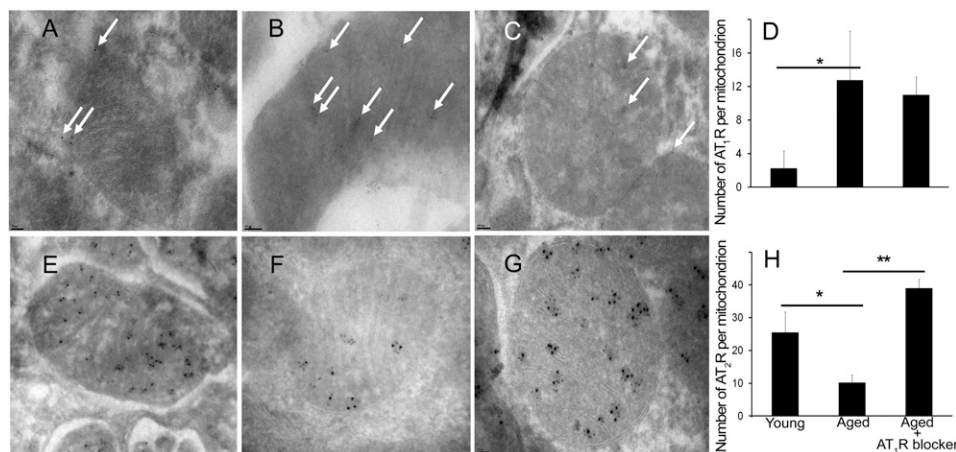


Fig. 7. Effect of AT₁R blocker losartan on expression of mitochondrial AT₁Rs and AT₂Rs. Renal tubular cell sections from C57BL/6 20 wks old (A and E), 70 wks old (B and F), and 70 wks old treated with losartan 40-60 mg/kg/day for 20 wks (C and G). D and H represent average counts of immune-labeling densities of mtAT₁R (D) and mtAT₂R (H) by age group in response to losartan. Gold particles in 30 mitochondria from each immunolabeling experiment were counted and averaged. Age was associated with a significant decrease in mtAT₂R that was reversed with chronic use of losartan. (* $P < 0.005$, ** $P < 0.0005$.)

Immunolocalization of AT₁R, AT₂R, and Ang II. Immunogold electron microscopy was performed as described (48). The sections were probed against AT₂R, Ang, or AT₁R antibodies.

Mitochondrial Respiration. Isolated rat heart or liver mitochondria were aliquoted into a XF96 microplate (Seahorse Bioscience) (49). AT₂R agonist and antagonist and an arginine analog that inhibits NO production (L-NAME) were added individually or combined, and oxygen consumption rates were determined by using a compartment model-based algorithm.

Mitochondrial Membrane Potential Measurements. Changes in isolated mitochondria membrane potential ($\Delta\Psi_m$) were quantified from changes in tetramethylrhodamine methyl ester (TMRM)-positive fluorescence in response to increasing concentrations of AT₂R agonist CGP421140 being added to the mixture (50).

Human and Animal Groups. Mitochondria were derived from young adult humans (20–30 y old), adult rats (24 wk old), adult (20 wk old) and aged (70 wk old) mice, and a human cell line. Adult mice at 50 wk of age were treated with the AT₁R blocker losartan at doses of 40–60 mg/kg per day for 20 wk.

Statistical Analyses. Data are expressed as means \pm SD and analyzed with the one-way ANOVA program. Differences were considered significant at $P < 0.05$.

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