LESSONS FROM IN VITRO STUDIES AND A RELATED INTRACELLULAR ANGIOTENSIN II TRANSGENIC MOUSE MODEL

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Cook JL, Re RN. Lessons from in vitro studies and a related intracellular angiotensin II transgenic mouse model. Am J Physiol Regul Integr Comp Physiol 302: R482–R493, 2012. First published December 14, 2011; doi:10.1152/ajpregu.00493.2011.—In the classical renin-angiotensin system, circulating ANG II mediates growth stimulatory and hemodynamic effects through the plasma membrane ANG II type I receptor, AT1. ANG II also exists in the intracellular space in some native cells, and tissues and can be upregulated in diseases, including hypertension and diabetes. Moreover, intracellular AT1 receptors can be found associated with endosomes, nuclei, and mitochondria. Intracellular ANG II can function in a canonical fashion through the native receptor and also in a noncanonical fashion through interaction with alternative proteins. Likewise, the receptor and proteolytic fragments of the receptor can function independently of ANG II. Participation of the receptor and ligand in alternative intracellular pathways may serve to amplify events that are initiated at the plasma membrane. We review historical and current literature relevant to ANG II, compared with other intracines, in tissue culture and transgenic models. In particular, we describe a new transgenic mouse model, which demonstrates that intracellular ANG II is linked to high blood pressure. Appreciation of the diverse, pleiotropic intracellular effects of components of the renin-angiotensin system should lead to alternative disease treatment targets and new therapies.

intracrine; mitochondria; nuclear intracrine

THE RENIN-ANGIOTENSIN SYSTEM is a phylogenetically ancient hormonal pathway, existing as a circulating system and serving pressor functions, even in primitive vertebrate cyclostomes, such as the river lamprey (127). In recent times, it has become clear that angiotensin can accumulate in various mammalian tissues and organs, including kidney (75, 100, 123), pancreas (22, 23, 86, 95), heart (78, 118, 144), and brain (89, 136, 165) independently of the circulating system and can mediate local effects. In both the circulating and local systems, the canonical effects of ANG II are mediated through the ANG II type 1 (AT1R) and type 2 (AT2R) G protein-coupled receptors. AT1R is the predominant receptor in adult animals and that which mediates contractile, pressor, secretory, and growth effects. AT2R, which shares only about 30% identity with AT1R, in contrast, may play a role in abrogating vasoconstrictor and growth responses to ANG II-mediated AT1R activation (21, 146, 147). AT1R is typically coupled through Gαq/11 to the second messengers 1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which, in turn, activate PKC and trigger release of intracellular calcium stores. AT1R can also be coupled to Ras, Raf-1, MAPK, phospholipase A, JAK-STAT, and Jun kinase pathways, among others (68, 103, 149, 170). The AT1R is also reciprocally coupled to a number of receptor tyrosine kinases (RTKs), including EGF receptor and insulin-like growth factor-1 (117, 154). AT1R transactivates RTKs, thus stimulating ERK1/2 phosphorylation activation in hepatic cells, vascular smooth muscle cells (VSMC), and breast cancer cells (36, 113, 117, 138). This pathway appears to involve sequential activation of the AT1R, Gαq/11, PLC, and Src. The protein tyrosine kinase Src, in turn, activates a matrix metalloprotease [ADAM17 in the case of VSMCs (116)]. The matrix metalloprotease catalyzes cleavage and shedding of heparin-binding EGF, which activates the EGF receptor (141). Src may also directly bind and activate the receptor. Clearly then, ligand stimulation of AT1R at the plasma membrane influences multiple pathways, the collective outcome depending on cell type and environment.

Functional Intracellular AT1 Receptor

... In the cytoplasm. For the G protein-coupled receptors (GPCRs), binding of agonist sequentially activates multiple G protein assemblies until the receptor desensitizes to agonist.
Desensitization generally involves receptor phosphorylation by G protein-coupled receptor kinases, leading to enlistment of the β-arrestin adaptor protein and targeting the receptor for internalization into clathrin-coated pits. Classical GPCR signaling ceases following internalization into clathrin-coated pits. However, signaling can continue through an alternate G protein-independent β-arrestin-dependent pathway. β-arrestin can act as a scaffolding protein to link the AT1R to other signaling pathways, including p38 MAPK, ERK1/2, and JNK3 (c-Jun N-terminal kinase 3) (25, 63, 93, 142). While β-arrestin proteins were traditionally known for reducing receptor signals, they can also initiate signals in the cytoplasm from the same receptors that they “desensitize” at the plasma membrane (142).

Ligand stimulation of AT1R can activate ERK1/2 directly through Goq/11 or indirectly through a β-arrestin-mediated signaling pathway. G-protein-mediated AT1R activation of ERK is rapid, transient, and blocked by PKC inhibitors, and results in nuclear translocation of phospho-ERK. In contrast, the β-arrestin pathway is slower to commence and is characterized by persistent activated ERK associated with endocytic vesicles. Clearly, AT1R retains a function, albeit a static function, following internalization into cells. This permits signaling to continue “after receptor internalization” into endosomes. We believe this signaling pathway coincides with the large endosomes that have been described by Hunyady et al. (67) and Innamorati et al. (71) as the Rab11-positive perinuclear recycling compartment. Hunyady et al. (67) describe two pathways by which the AT1R may traffic. One is the Rab4-positive short recycling pathway, which leads to rapid recycling of the receptor to the cell surface. The other is the Rab11-positive long recycling pathway, in which the AT1R collects in endosomes of the perinuclear recycling compartment (PNRC). In a similar fashion, Shenoy and Lefkowitz (142) suggest that GPCRs can be categorized as class A (primarily recycled rapidly, and includes β2-adrenergic, ETA, and the μ-opioid receptor), receptors of which preferentially bind β-arrestin 1 and dissociate rapidly. Class B GPCRs (which include AT1R, neurotensin I, and TRHR) show equal affinity for β-arrestin 1 and β-arrestin 2 (142). The Class B GPCRs form stable complexes with and traffic together with arrestins, and they colocalize in endosomes for extended periods of time. In the absence of evidence to the contrary, we believe the arrestin-AT1R (class B) complexes, which are stable and continue signaling for significant periods of time, reside in the endosomes, described by Hunyady et al. (67) as the long recycling pathway, or PNRC.

We have also found that this compartment shares membranes with the Golgi apparatus (30), suggesting that materials could be retrotransported via the PNRC endosomes.

... At the nuclear membrane. A number of prototypical GPCRs, including the type I lysophosphatidic acid (LPA) GPCR (LPA1), and the β-adrenergic receptor, exist as holoproteins in the nuclear membrane and possess nuclear functions. LPA1 is both constitutive in nucleus and traffics to nucleus in response to LPA treatment, and stimulates phosphorylation of intranuclear proteins, including the Akt kinase protein (52, 164). Moreover, LPA mediates, through the LPA1 receptor, eNOS translocation to perinuclear and nuclear sites. eNOS through nitric oxide, in turn, modulates calcium homeostasis and gene transcription (53). Similarly, β1- and β3-adrenergic receptors are present on nuclei of ventricular cardiomyocytes, bind ligand, and stimulate downstream effects, including adenyl cyclase activity and MAPK activation (15, 159).

The AT1 receptor and the ANG-(1–7) receptor both localize within the nuclear membrane (30, 62, 111). Because the nuclear double-membrane is continuous with the endoplasmic reticulum (ER), receptors can flow freely between the two compartments (see Fig. 1). The diffusion-retention model for nuclear trafficking predicts that transmembrane or integral membrane proteins in the ER can diffuse laterally in a retro-

![Fig. 1. Illustration of the nuclear envelope cross section. Receptors from the ER can traffic through the outer nuclear membrane, pore membrane domain, and into the inner nuclear membrane. Receptors can be maintained in the inner membrane by attachments to the lamina or chromatin. Figure was reproduced with kind permission from Springer Science+Business Media B.V. from The Local Cardiac Renin-Angiotensin Aldosterone System, 2nd ed., 2009, Chapter 4, “Intracellular Accumulation and Nuclear Trafficking of Angiotensin II and the Angiotensin II Type I Receptor,” Cook and Re, Fig. 4.1a, p. 31. [From Cook and Re (30).]
grade direction from the ER through the outer nuclear membrane and then through the lateral channels of the nuclear pore complexes and into the inner nuclear membrane (150, 166). This model predicts that proteins collect in the inner nuclear membrane at higher steady-state levels than in other compartments based on an association with resident proteins, which immobilize the diffusing species. Full-length GPCRs, such as AT1R, therefore, can accumulate in the inner nuclear membrane by retrograde passage from the ER (30). While Golgi modifications are not required for function of many GPCRs, including the AT1R (88), some nuclear membrane-associated receptors do appear to be glycosylated, suggesting that they may traffic to the ER from the Golgi via retrograde COPI vesicles (5) and then accumulate in the nuclear membrane via lateral diffusion from the ER.

Such receptors have potential to interact with ligands present in the internuclear membrane space and to signal events in the nucleus through nuclear membrane signal transduction events (51) that may recapitulate plasma membrane events. For nuclear membrane-associated AT1R, the ligand binding site presumably exists in the internuclear membrane space (INMS). ANG II, therefore, must be present in the INMS to activate the receptor. In isolated nuclei studies, ANG II can enter the INMS via disruptions in the outer membrane or in the ER-nuclear membrane interface that are caused by the nuclear isolation technique. The availability of the receptor canonical binding site to ANG II should be dependent on the quality of the nuclei and contiguity of inner and outer nuclear membranes (79, 80). How might ANG II reach its nuclear membrane-associated receptor target in an intact cell? We suggest that ANG II can only gain access to the INMS through intercompartment membrane fusions (since the nuclear membrane-ER is a closed membrane system). A candidate population for vesicular delivery of ANG II is the endosomes of the perinuclear recycling compartment. We have shown (as have others) that AT1R localizes to these vesicles following internalization. We have further shown that these endosomes colocalize with Golgi (30). This fusion would, in theory, permit retrograde trafficking of ANG II to the INMS via COPI vesicles (5). This would also represent a mechanism for trafficking of modified (Golgi-processed) AT1R in a retrograde manner into the inner nuclear membrane.

Several studies have shown directly that the endothelin (ETB but not ET_{A}) receptor and AT1R are present in both nuclear membranes and nucleosol (11, 13, 14, 30) and are directly activated to increase nuclear free calcium, suggesting that they are functional receptors. The fact that the corresponding ligands can be found within the nucleus as well, suggests that ligand-receptor interactions, which recapitulate those found at the plasma membrane, may exist at the nuclear membrane-nucleosol interface. Chappell and colleagues (61, 119, 120) have characterized AT1 nuclear membrane receptors in rat and in sheep kidney. They find both that ANG II upregulates reactive oxygen species in isolated renal nuclei through AT1 receptors and that nuclear AT2 receptors are functionally linked to nitric oxide production. In both fetal and adult sheep, most cortical nuclear and plasma membrane sites are AT2 receptor-like, while most medullary nuclear and plasma membrane sites correspond to AT1 receptors.

Several reports point to the existence and induction of phosphatidylinositol diphosphate (PIP), IP3, DAG, phospho-

lipase C (PLC), and PKC in the nuclear compartment of many cell types, including 293, PC12, and NIH 3T3 (72, 73, 109, 152). For example, cellular treatment of human osteosarcoma cells with IL-1α induces intranuclear PIP and PLC (174), while treatment of 3T3 cells with IGF-1 induces enhanced nuclear DAG levels (106). Moreover, several cell types, including those which possess nuclear AT1, ET1, and neuroepithelium Y GPCRs (including heart, hepatic, vascular endothelial, and smooth muscle cells) also possess nuclear membrane AT2 receptors. The data supporting the concept of nuclear signaling cascades that recapitulate plasma membrane cascades are abundant and support the dogma that the nucleus represents a modified “cell within a cell.”

Intracellular receptor fragments. A number of GPCRs, including the vasopressin V2, β2 adrenergic, and ET1 receptors, are reported to undergo regulated limited proteolysis to produce peptides with possible bioactivity (57, 58, 60, 83, 84). For most GPCRs, however, it is unclear whether an intracellular fragment (as compared with an ectodomain fragment) is also generated during proteolysis, generally because the appropriate assays have not yet been performed. In addition, several GPCRs have been identified, associated with cellular nuclei, including those for ACh, ANG II, apelin, dynorphin B, endothelin 1, and prostaglandin E2, and often using multiple different approaches (51, 92). For instance, the AT1 receptor has been localized to nuclei in several different independent studies using techniques that include radioligand binding and chromatin solubilization assays of rat liver nuclei, immunohistochemistry of rat brain, electrophysiology assays of rat cardiac myocytes, ANG II microinjection and calcium assays, immunochemistry, and Western blot of rat brain neurons, and immunocytochemistry and Western blot of human VSMCs (28, 51, 129, 131). In these nuclear association studies, assays have not generally been designed to differentiate between cleaved receptor fragments and holoreceptors.

In tissue culture studies, we have specifically addressed the nature of the intranuclear AT1 receptor using a double-fusion protein of AT1R, in which yellow fluorescent protein is fused downstream and cyan fluorescent protein (CFP) is fused upstream. We have shown that the fluor colocalize in vehicle-treated transfected cells, whereas the fluor diverge in ANG II-treated transfected cells (28). The receptor, therefore, is cleaved in a ligand-dependent manner. Cyan fluorescence is lost from the cell surface (seen as a reduction in blue fluorescence at the circumference), while yellow fluorescence accumulates in nuclei. Similar results are obtained when alternate tags (myc downstream and Flag upstream) are substituted for the fluorescent moieties. The cleavage occurs in genetically unmodified protein as well, releasing a stable 6-kDa protein within cells (28, 30, 32). The cleavage fragment (CF or AT1{\textsubscript{R}CF}) does possess a nuclear localization signal, and mutation of the consensus reduces, but does not inhibit, nuclear localization (32). Active transport may occur to increase accumulation of the CF, but the size of the cleavage fragment should permit passive diffusion into the nucleus through nuclear pores (76, 77).

For comparison, an intracellular fragment is also produced from the GPCR, D-frizzled 2, a receptor for the ligand, wingless (or Wnt in mammals), which is involved in pattern devel-
opment in Drosophila and mammals. The intracellular domain of D-frizzled 2 is cleaved from endosomes and translocated to the nucleus where it is involved in transcriptional events that support pattern formation (108). The carboxy terminus of Frizzled-7 is also cleaved, although the fate of the resulting intracellular fragment has not yet been reported (151) Furthermore, an intracellular cleavage fragment is produced from the carboxy terminus of polycystin, a noncanonical GPCR having 11 transmembrane domains (9, 101). Following nuclear localization, the carboxy-terminal fragment inhibits the ability of β-catenin to induce T-cell factor-dependent gene transcription and, thereby, inhibits the Wnt signaling pathway (87). The detailed processes and enzymes responsible for cleavage of most multipass receptors are not clear, although a family of intramembrane proteases referred to as IMPAS/PSH/signal peptidase (or SPP) is implicated (112). Collectively, these studies indicate that cleavage of receptors and other cell surface proteins, as well as accumulation of stable intracellular products, can be regulated processes, which serve, perhaps, to further amplify or enhance effects of ligand-receptor signal transduction events, which initiate at the plasma membrane.

We have recently determined the precise cleavage site within the AT1R by mass spectrometry and Edman sequencing (32). Cleavage occurs between Leu508 and Gly509 at the junction of the 7th transmembrane domain and the intracellular cytoplasmic carboxy-terminal domain. To evaluate the function of the CF distinct from the holoreceptor, we generated a construct encoding the CF as an in-frame yellow fluorescent protein fusion. The CF accumulates in nuclei and induces apoptosis, as determined by nuclear fragmentation and disintegration, phosphorylserine displacement in the plasma membrane caspase activation, poly(ADP-ribose) polymerase upregulation, and terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) labeling and DNA fragmentation. CF-induced apoptosis appears to be a general phenomenon, as it is observed in multiple cell types, including smooth muscle cells and cardiomyoblasts.

Interestingly, many intracellular cleavage products correlate with cell death and apoptosis, consistent with the action of AT1RCF. For example, the receptor for advanced glycation end products (RAGE) has been linked to several chronic diseases thought to result from vascular damage, including atherosclerosis, peripheral vascular disease, Alzheimer’s disease, and congestive heart failure. RAGE is targeted by regulated intramembrane proteolysis, producing both an extracellular soluble fragment (sRAGE), as well as an intracellular domain; the intracellular protein is detected in both the cytoplasm and nucleus (50). Transfected human embryonic kidney 293 cells that exhibit accumulation of this product in the nucleus also show nuclear condensation and cell shrinkage. This is accompanied by a 16% and 38% reduction in cell viability at 16 and 40 h posttransfection, respectively, and also in an increase in TUNEL-positive cells at 16 h posttransfection.

Regulated intramembrane proteolysis is also involved in the pathogenesis of Alzheimer’s disease through a pathway distinct from RAGE. The transmembrane amyloid precursor protein (APP) gives rise to the β peptide cleavage product, which is found in plaque fibrils and tangles (4). The APP also gives rise to an APP intracellular domain (AICD), which translocates to the nucleus and appears to contribute to the pathogenesis of Alzheimer’s disease, perhaps by regulating nuclear signaling (160). Recent studies have shown that overexpression of the AICD in neurons induces cell death, as determined by TUNEL assays and DNA laddering (115), possibly in collaboration with Fe65 and p53. Another example of cleavage fragment-induced apoptosis occurs in a family of receptors that are involved both in internalization of ligands and also in signal transduction and neurotransmission. Cleavage of both the low-density lipoprotein receptor-related protein (LRP), as well as the related LRPI contributes to apoptosis. LRP undergoes regulated intramembrane proteolysis in response to ischemia in neurons with nuclear translocation of the intracellular domain. The latter induces caspase-3 cleavage, TUNEL positivity, and significant cell death (124).

Clearly then, other receptor cleavage fragments, like the AT1RCF, have been associated with nuclear transport and apoptosis. An underlying homology in the sequences of the cleaved peptides, however, is not readily apparent. Nor is there any unambiguous reason why regulated proteolysis of these particular diverse receptors might be linked to cell death. Further investigation of the caspase pathways activated by the AT1RCF may be helpful in formulating a thesis.

Functional Intracellular Angiotensin

Studies have been reported since the 1970s that support the existence and functionality of intracellular or intracrine angiotensin II (iANG II). These studies originate in many laboratories and include a broad spectrum of models and techniques that can be collected into distinct bodies of work that support the following ideas: 1) exogenous ANG II associates with cellular nuclei and other subcellular organelles from rat heart and liver (3, 18, 66, 130, 132–134, 136–138, 159, 160), 2) endogenous iANG II exists in distinct cells and tissues, such as rat juxtaglomerular cells, proximal tubule cells, and brain [including anterior pituitary, subfornical organ, hypothalamus, and third ventricle (2, 45, 70, 110, 135, 153, 161, 172, 173)], 3) ANG II can be detected in nuclei of native cells in rat cerebellar cortex, human endocardial endothelial cells, rat renal cortex, and porcine kidney and adrenals (45, 49, 77, 100, 158), 4) introduced (microinjected or genetically expressed) ANG II possesses intracellular function in, among other tissues, rat hepatoma and VSMCs, hamster ventricular cardiac myocytes, rabbit proximal tubule cells, and transfected cells, including CHO-K1 and COS-7 cells (26, 27, 29, 31–33, 37, 39, 40, 173), and 5) ANG II stimulation can cause AT1R translocation to nucleus in human and rat VSMCs, CHO-K1, and COS-7 cells, and rat brain neurons (14, 27–30, 102).

Intracellular ANG II from intracellular angiotensinogen. To investigate the potential for (and subsequent effect of) ANG II generation within cells, we mutated an angiotensinogen (AGT) cDNA to remove the signal peptide required for secretion through the secretory pathway. We ligated it into an expression plasmid to produce a nonsecreted form of AGT in transfected cells and confirmed that ANG II is, indeed, expressed and retained in cells following transfection. ANG II produced from this construct is generated through an intracrine mechanism. We investigated the effect of this plasmid, following transient and stable transfections and in the presence of various effectors, upon cellular proliferation (33). Proliferation of rat hepatoma cell lines was increased an average of 39%, growth that was not affected by the AT1R.
blocker candesartan (which acts exogenously) but was blocked by renin antisense phosphorothioate oligomers, suggesting that growth is stimulated through a renin-dependent pathway. This study showed that ANG II can be processed from AGT, which is retained within cells. Therefore, assuming that AGT can be internalized and accumulate in native cells and tissues or assuming that a nonsecreted form of AGT might be generated and retained in cells, there is potential for ANG II intracellular generation in native tissues (30).

A number of other hormones and growth factors, including prolactin, insulin, nerve growth factor, epidermal growth factor, somatostatin, neuropeptide Y, and platelet-derived growth factor have been found to have biologically significant intracellular activities (17, 41, 46, 54, 85, 126, 128, 130). Clearly, where growth factors have dual extracellular and intracellular roles, they may be 1) secreted locally and internalized or 2) processed from a precursor (where applicable) and retained with the cell. Hepatoma-derived growth factor (HDGF), a heparin-binding growth factor, similar to the fibroblast growth factors, functions through the latter mechanism. It is a secreted growth factor ubiquitously expressed and mitogenic for fibroblasts and endothelial cells (46, 114). However, HDGF is also present in nuclei (its sequence contains a putative nuclear localization element) of a variety of cell types and is involved in proliferation during development and disease (46, 114). Following transfection of hemagglutinin-tagged HDGF, the tag is observed in scattered isolated nuclei, not in cell clusters, suggesting that it directly translocates to the nucleus and is not locally secreted, internalized, and subsequently translocated to the nucleus. HDGF can reside in the cytoplasm or nucleus, or be secreted, depending on the cell type and cell-cycle phase, and nuclear HDGF has been associated with hepatocellular carcinoma, pancreatic cancer, colorectal stromal tumors, and nasopharyngeal carcinoma (65, 66, 94, 155, 162).

Consistent with evidence that elevation of intracellular HDGF may directly reflect the disease state of a cell, iANG II in myocardium (myocytes and endothelial cells) of diabetic rats and diabetic hypertensive patients may similarly reflect disease severity. In rat models, high glucose stimulates elevation of intracellular ANG II, oxidative stress, and fibrosis (145). In human diabetic patients, intracellular ANG II levels in hearts are increased 3.4- and 3.1-fold, respectively, in myocytes and endothelial cells over levels present in nondiabetic patients. Intracellular ANG II levels appear to be increased an additional twofold in diabetic hypertensive patients compared with diabetic nonhypertensive patients (48). The authors suggest that local elevations of ANG II, which accompany diabetes and hypertensive diabetes, in turn, enhance oxidative damage and activate cardiac cell apoptosis and necrosis. Elevated iANG II may, therefore, be both a marker of disease and a contributor to disease progression.

In some cases, internal production or internalization of a peptide or protein has been proven to mediate very different effects from those resulting from interaction with cell surface receptors. For example, parathyroid hormone-related protein (PTHrP) acting within nuclei of vascular smooth muscle cells stimulates cell division and contributes to growth of breast and colon cancers, whereas extracellular PTHrP inhibits proliferation (10, 85, 107). The PDGF family of proteins provides an additional example. Binding of the v-sis or PDGF B proteins to the (underglycosylated) 160-kDa PDGF receptor within the secretory compartment elicits a transforming signal that is not reproduced by v-sis or PDGF B binding to the mature 180-kDa receptor on the cell surface (7, 8, 18). Ligand-receptor binding within the secretory compartment is both necessary and sufficient for v-sis-mediated transformation. Neither v-sis nor PDGF-B (which possesses 94% amino acid homology to v-sis) can transform immortalized cell lines by binding surface PDGF receptors. In addition to the well-characterized PDGF external autocrine loop and internal autocrine (intracrine) loop involving receptor activation within the processing compartments, PDGF may have a third autocrine function. v-Sis has been localized to the nucleus (as well as polyribosomes, endoplasmic reticulum, and Golgi apparatus) of simian sarcoma virus-transformed fibroblasts (122, 171). Indeed, prominent immunoreactive proteins have been found in association with nuclear chromatin in intact cells and in isolated nuclei. Nuclear localization signals have also been mapped to v-sis, PDGF B, and PDGF A protein products (91, 105), suggesting that PDGF might be transported to the nucleus following binding and internalization. PDGF is an example of a growth factor that appears to function at multiple sites and through multiple pathways to achieve its regulatory effects. We believe that ANG II in a similar fashion may act at the cell surface or within the nucleus or mitochondria and that it may be internalized or generated internally, depending upon cell type, cell age, genetic background, and cellular environment (or culture conditions).

In conclusion, these studies support the hypothesis that angiotensin and a growing list of additional peptides, growth factors, and cytokines can generate biologically relevant effects by acting at intracellular sites. At least some of these events follow retention and action of the intracrine in the cell of synthesis.

Internalized extracellular ANG II. The existence of an intracellular renin-angiotensin system (iRAS) implies that components of the RAS are made locally and result in biologically functional intracellular angiotensins, renin, and/or receptor. Studies show that measurable levels of ANG II exist within some cells and that ANG II may be released from certain cell types (e.g., cardiac myocytes and mesangial cells) following mechanical stimuli, such as stretching (6, 38, 64, 69, 134, 139, 157). From where does this intracellular ANG II originate? Existing intracellular ANG II may be internalized from the circulation or extracellular fluid, or alternatively, produced intracellularly.

One model for the accumulation of iANG II relies on the knowledge that ANG II, within ligand-receptor complexes, is internalized via clathrin-coated pit-mediated endocytosis. Because it is well known that contents of endosomes can leak into the cytoplasm (19, 20, 24), ANG II may directly discharge into the cytosol and could access binding sites within the cytosol and nucleosol (the latter via nuclear pores). Several studies show that megalin may play a role in internalization of AGT and ANG II in the proximal nephron. Megalin is a multiligand scavenger receptor involved in protein endocytosis. Studies suggest that in several cell types, including yolk sac epithelium and proximal tubule (brush border membrane vesicles), megalin can bind to and internalize ANG II and ANG 1–7 (55, 56). In more recent studies, megalin was also found to internalize AGT in the early proximal tubule (123). The favored model is that AT1R-mediated ANG II uptake favors iANG II accumu-
loration, whereas megalin-mediated ANG II uptake favors iANGII degradation, but megalin could, in theory, mediate ANG II transport to intracellular sites.

To our collective knowledge, few studies have been designed to address ANG II transport to the internuclear membrane space. We have proposed and have conducted some preliminary studies to support the idea that ANG II may reach the internuclear membrane space by way of recycling endosomes (30) (see subsection . . . In the cytoplasm).

Transgenic Mouse Models

The functions of renin, angiotensin, ACE, and RAS receptors has been extensively studied in transgenic models (47, 59, 82, 96, 98, 135). Moreover, the role of intracellular renin has also been evaluated in several transgenic studies (90, 97, 121, 167, 168). While intracellular angiotensin has been broadly studied in cell culture and in vivo following adenosiviral delivery (3, 97), we recently reported the first study describing a transgenic mouse model, which directly expresses intracellular ANG II, independent of secreted AGT or secreted ANG II (133). Our approach was based on results from our earlier in vitro studies of a protein fusion of angiotensin, in which ANG II is fused downstream and in-frame with enhanced cyan fluorescent protein (ECFP/ANG II) (with a 10-amino acid spacer arm between the two moieties). Following transfection, ANG II in the context of this fusion is detectable within cells using anti-ANG II antibodies, by Western blot, and by enzyme immunoassay. ECFP/ANG II is shown to remain intact and not degraded by Western blot analysis. In tissue culture studies, we found iANG II to alter the steady-state distribution of fluorescently labeled AT1R with receptor translocating to the nucleus of COS-7, CHO-K1, and rat vascular smooth muscle cells. We also found ANG II to increase proliferation and activate CREB in all of these cell types. Moreover, we confirmed that ECFP/ANG II is maintained within cells (not released) and activates some signal transduction pathways uniquely different from exogenous ANG II signaling. These studies prompted us to generate a transgenic mouse line overexpressing ECFP/ANG II (133). We selected the metallothionein promoter to drive expression to a wide array of tissues with the potential for metal regulation of transgene expression. While transgene is expressed (RNA and protein) in all major tissues in two independent mouse lines, kidney is the only tissue in which we detect a phenotypic change. As early as 2 mo of age, these mice demonstrate elevated systolic and diastolic blood pressures, as determined by radiotelemetry (Fig. 2) (133). Some mice also display kidney thrombotic microangiopathy (TMA) and microthrombosis in the glomerular capillaries and small vessels. TMA has been observed in other transgenic models that overexpress components of the RAS (104, 140). iANG II-mediated changes in blood pressure (BP) are observed as early as 2 mo of age. TMA, which follows at 4–6 mo of age (and ranges from very mild to severe), is not, therefore, responsible for elevated BP in this model. We observe no sex-specific effects of ECFP/ANG II on BP or TMA; males and females are affected to the same extent by the transgene. Circulating ANG II can, in contrast, cause sex-specific effects. Elevation of circulating ANG II, as occurs in rodent infusion of ANG II, can cause sexually dimorphic changes in BP that have been linked to gonadal hormones, baroreflex control of HR/BP, and sympathetic nerve activity (169). We are not aware of any reports in which intracellular ANG II or receptor show sex-specific differences in distribution or function. However, because iANG II can act at a number of divergent locations in the cell and through a number of distinct mechanisms, some of the intracellular effects could potentially be sex-specific. Studies have shown that ANG II infusion into mice stimulates NAD(P)H oxidase and reactive oxygen species (ROS) species formation in a sex-specific fashion (44, 156). There also exists evidence that ANG II treatment of isolated nuclei increases NAD(P)H oxidase activity and ROS formation through an AT1R pathway (120), suggesting that some nuclear iANG events might also be sex-specific.

Analysis of isolated mouse embryonic fibroblasts (MEFs) from transgenic mice shows the presence of ECFP/ANG II in the cytoplasm and in some (30–40%) nuclei. The cytoplasmic fluorescence is often punctate and significantly colocalizes with mitochondrial markers, suggesting a mitochondrial function (31, 132). Our studies and those from other laboratories have demonstrated ANG II and ANG II binding sites associated with mitochondria of MEFs, kidney, adrenals, and brain (45, 132, 148, 158), and mitochondria have been implicated in RAS-mediated disease, including vascular endothelial dysfunction, hypertension, and diabetes (31, 34, 35, 42, 43, 132). Unquestionably, mitochondria play a role in RAS-mediated disease. It remains to be determined whether and to what extent ANG II mediates its mitochondrial effects through canonical (membrane-associated receptor) vs. noncanonical means. Early studies from our laboratory indicate a direct interaction between iANG II and mitochondrial electron transport chain components, as well as effects on ATP generation and the formation of ROS. Direct binding of iANG II to mitochondrial proteins would preclude binding to the binding pocket of integral membrane AT1R. These data, therefore, suggest a noncanonical action of ANG II at mitochondria (31, 132). The
fact that intracellular ANG II also increases blood pressure in
our transgenic model suggests that noncanonical ANG II action
at mitochondria plays a role in ROS generation and hyperten-
sion.

Abadir et al. (1) have recently presented compelling
evidence for the existence of functional intramitochondrial
angiotensin receptor. They evaluated several cell types (in-
cluding mouse cardiomyocytes and renal tubule, vascular
endothelial, and neuronal cells) and showed the presence of
AT2R in all of these using Immunogold staining. AT1R was
typically present only very rarely in 5-mo-old mice but
increased in prevalence in aged mice (greater than 1 yr of
age). For example, in proximal tubules, this paper reports an
average of two occurrences of AT1R per mitochondrion,
(increasing to 12 molecules in aged mice) and ~25 copies of
AT2R per mitochondria, that number falling to 10 in aged
mice. Moreover, they show mitochondrial AT2R to be
coupled to increased mitochondrial nitric oxide production,
suggesting a link between AT2R and mitochondrial aging in
disease. These data support a canonical role for angiotensin
receptors in mitochondria.

Collectively, the published reports suggest that ANG II may
be internalized or generated through an intracellular system
and that it may alter cellular properties both through cytoplas-
mic protein interactions and through nuclear translocation,
receptor binding, and transcriptional regulation of gene expres-
sion (Fig. 3). iANG II that is both internalized through recep-
tor-mediated endocytosis and potentially processed from intra-
cellular AGT may have noncanonical functions in the cyto-
plasm or nucleosol or may function in a canonical fashion
through nuclear membrane-associated receptor. Moreover,
ANG II may act at the level of the mitochondria through
canonical (mitochondrial membrane-associated receptor) or
noncanonical mechanisms. Most importantly, iANG II alters
cellular proliferation and signal transduction and elevates
blood pressure through a renal mechanism in transgenic mice.
Furthermore, the AT1 receptor can function independently
of ANG II, participating in a cytoplasmic β-arrestin-mediated
scaffold for signaling events. Moreover, cleavage fragments
of the receptor can traffic to the nucleus and induce apoptosis.

Perspectives and Significance

Fig. 3. Model of pathways through which the iRAS may mediate intracellular effects. [1] Endosomes containing embedded AT1R can be internalized and AT1R
can continue to signal through the β-arrestin:AT1R complex which serves as a scaffold for assembly of ERK and JNK signaling components (142). [2] Acidification of endosomes (20, 24) permits release of ANG II into the cytoplasm, where it may traffic to nucleus [3] or mitochondria [4], either free or bound
to other proteins. Cytosolic ANG II can mediate signaling effects by modifying protein complex activity, or it can be transported into the nucleus as a complex
or as a free peptide. In the nucleus, association of ANG II with other proteins, including, potentially, a nuclear form of ANG II receptor may permit modification
of gene expression or DNA replication. In addition, a cleaved fragment of the AT1R (COOH terminus) (28) [5, 6] may associate with a protein complex that
includes ANG II [7] and traffic to nucleus [8]. AT1R can accumulate in nuclear membrane (11, 30) presumably by retrograde membrane diffusion from the
endoplasmic reticulum (ER)/Golgi apparatus. Receptor associated with the inner nuclear membrane [by movement from the outer nuclear membrane around the
nuclear pore complex (137, 166)] is positioned such that the COOH terminus is within the nucleosol [9] and available for signaling through nuclear second
 messenger signaling pathways (11, 12, 16, 81, 125). Presumably, nuclear signaling through nuclear membrane-associated AT1R is ligand mediated. We believe
that ANG II may gain access to the ER lumen and, subsequently, to the intranuclear membrane space, via trafficking through the slow recycling endosome
pathway (30, 67). [10] In addition, ANG II may be generated within cells from intracellular AGT (30, 143).
Clearly, the RAS is proving to be far more complex than could have been predicted a mere decade ago and contributes to the principle that biological systems are inherently efficient, often showing signal amplification in successive steps of a given pathway, with reutilization and minimal waste. Although we know that signal transduction contributes to the specificity of ligand-mediated responses, it more importantly contributes to amplification of the response. We argue that the continued signaling that occurs in intracrine systems and through non-classical intracellular receptor functions represents an extension of the amplification principle, permitting increased magnitude and/or duration of response.

The development of new drugs directed to noncanonical functions of intracines and atypical intracellular receptors and receptor fragments represents a new pharmaceutical industry interest. Most GPCR-modulating drugs on the market were not initially targeted to a specific protein but were developed on the basis of a functional activity assay. The observation that a candidate drug activated or inhibited a GPCR, in most cases, was only later discovered. GPCR-targeted drugs typically inhibit cell surface receptors and are often not specifically designed to be efficiently internalized into cells. Moreover, they are not designed to target noncanonical intracrine functions. Effective targeting of nonclassical functions of GPCRs and targeting of cleaved fragments or intracellular domains generated from plasma membrane proteins will, in most cases, require novel strategies.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.L.C. interpreted results of experiments; J.L.C. prepared figures; J.L.C. drafted manuscript; J.L.C. and R.N.R. edited and revised manuscript; J.L.C. and R.N.R. approved final version of manuscript.

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