Intracellular angiotensin II induces cell proliferation independent of AT₁ receptor

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Baker, Kenneth M., and Rajesh Kumar. Intracellular angiotensin II induces cell proliferation independent of AT₁ receptor. Am J Physiol Cell Physiol 291: C995–C1001, 2006.—We recently reported intracrine effects of angiotensin II (ANG II) on cardiac myocyte growth and hypertrophy that were not inhibited by the ANG II type 1 receptor (AT₁) antagonist, losartan. To further determine the role of AT₁ in intracrine effects, we studied the effect of intracellular ANG II (iANG II) on cell proliferation in native Chinese hamster ovary (CHO) cells and those stably transfected with AT₁ receptor (CHO-AT₁). CHO-AT₁, but not CHO cells, showed enhanced proliferation following exposure to extracellular ANG II (eANG II). However, when transiently transfected with an iANG II expression vector, both cell types showed significantly enhanced proliferation, compared with those transfected with a scrambled peptide. Losartan blocked eANG II-induced cell proliferation, but not that induced by iANG II. To further confirm these findings, CHO and CHO-AT₁ cells were stably transfected for iANG II expression (CHO-iA and CHO-AT₁-iA, respectively). Cells grown in serum-free medium were counted every 24 h, up to 72 h. CHO-iA and CHO-AT₁-iA cells showed a steeper growth curve compared with CHO and CHO-AT₁, respectively. These observations were confirmed by Wst-1 assay. The AT₁ receptor antagonists losartan, valsartan, telmisartan, and candesartan did not attenuate the faster growth rate of CHO-iA and CHO-AT₁-iA cells. eANG II showed an additional growth effect in CHO-AT₁-iA cells, which could be selectively blocked by losartan. These data demonstrate that intracrine ANG II can act independent of AT₁ receptors and suggest novel intracellular mechanisms of action for ANG II.

renin-angiotensin system; angiotensinogen; peptide hormones; nuclear signaling; intracrine

ANGIOTENSIN II (ANG II), the principal effector peptide of the renin-angiotensin system, has pleiotropic effects in multiple tissues (34). It has a central role in cardiovascular homeostasis and is implicated in the development of cardiac, vascular and renal pathologies. ANG II is formed in the circulation or locally in various tissues, through a cascade of reactions from components of local and/or systemic origin (12, 23). Thus, it acts as an endocrine, as well as an autocrine/paracrine peptide hormone. ANG II mediates effects through two types of receptors, AT₁ and AT₂, which are both seven-transmembrane G protein-coupled receptors (3). The AT₁ receptor accounts for the majority of the known functions of ANG II in various tissues. Binding of ANG II to AT₁ results in internalization of the AT₁-ANG II complex (2, 6, 43). While the receptor is recycled back to plasma membrane, ANG II is destined for intracellular locations such as lysosomes and the nucleus (14, 21, 44). The presence of ANG II binding sites on the nuclear membrane suggests a functional role for intracellular ANG II (4, 41). In addition to internalization in a complex with the receptor, the possibility of intracellular synthesis of ANG II has also been suggested by several studies (10, 32, 45). The existence of nuclear ANG II binding sites and intracellular ANG II (iANG II) strongly suggests a functional consequence. The possible clinical relevance of iANG II is particularly evident in the human diabetic condition, wherein intracellular staining for ANG II in heart is significantly increased compared with the nondiabetic state (18). We thus determined the functionality of iANG II and demonstrated that iANG II exerted biological, intracrine effects (1). We expressed ANG II intracellularly in myocytes, for both in vitro and in vivo studies. Overexpression of iANG II in cultured neonatal rat ventricular myocytes caused hypertrophic cellular growth. Cardiac-specific overexpression of iANG II in mouse resulted in biventricular cardiac hypertrophy. Significantly, the AT₁ receptor blocker losartan did not block the hypertrophic intracrine effects of iANG II. The lack of effect of losartan indicated that either losartan did not gain access to the intracellular compartment, or an AT₁-independent intracellular mechanism was operative. In this study, we determined the requirement for AT₁ receptors in the intracrine effects of ANG II. We used Chinese hamster ovary (CHO)-K1 cells as a model to address this question because these cells express little AT₁ receptor and do not respond to exogenous ANG II. These cells were stably transfected to express AT₁ receptor and iANG II. The growth properties of AT₁ and/or iANG II-expressing cells were compared with those of nonexpressing cells, to determine the requirement for AT₁ receptors in the intracrine effects of iANG II.

METHODS

Cell culture. CHO cells were cultured in Ham’s F-12K medium (Invitrogen) containing 10% FBS (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were transferred to serum- and antibiotic-free medium before performing the growth studies.

iANG II expression vector. The iANG II expression cassette (pcDNA/TO-iAng II) was similar to that which has been previously described for adenoviral expression of iANG II (1). Briefly, two complimentary oligonucleotides containing the coding sequence for the eight amino acids of ANG II, start and stop codons, and flanked by BamHI and XhoI sites (sense strand: 5'-GATCCCATTGACCCGGGTTACATCCACCCCTTTAAT-3'), were annealed and cloned into the pcDNA4/TO vector (Invitrogen). The coding sequence was flanked on the 5'-side by a complete cytomegalovirus enhancer.
promoter sequence, containing two copies of the tetracycline operator TetO 2 sequence, and by a 225 bp bovine growth hormone polyadenylation sequence on the 3’-side. A control vector was similarly generated, containing the scrambled ANG II peptide coding sequence (sense strand: 5’-GATCCATGAGCCACCGTATTTCAC-TCTAG-3’).

Transient transfection. Cells were seeded in 96-well plates at a density of 5 × 10^4 cells per well and grown overnight. Cells were transfected with the control or iANG II expression plasmid using Lipofectamine 2000 (Invitrogen) in serum-free medium for 4 h, followed by serum-containing medium overnight. The following day, transfected cells were transferred to serum-free medium or serum-free medium containing drugs, as described below.

Generation of stable cell lines. CHO cells stably transfected with rat AT1a wild-type receptor (CHO-AT1) were obtained from Dr. Junichi Sadoshima (The University of Medicine and Dentistry of New Jersey) (38). CHO and CHO-AT1 cells were stably transfected with pcDNA/TO-iANG II using Lipofectamine reagent. Stable clones (CHO-iA and CHO-AT1-iA, respectively) were selected by neomycin and zeocin resistance. Production of ANG II in cell lysates and in the medium of these cells was measured using a competitive ELISA, as previously described (36).

Radioligand binding assay. AT1 receptor expression was quantified by a competitive binding assay. Cells were seeded in 24-well plates at a density of 1.25 × 10^5 cells per well and grown overnight. Binding buffer [Dulbecco’s PBS with 2% BSA and 10 μg/ml bacitracin (Sigma)] containing 0.25 nmol/l [3-iodotyrosyl4-125I]ANG II (Perkin Elmer) and varying concentrations of unlabeled ANG II (0–1,000 nmol/l) was added to cells in a volume of 250 μl and incubated at 37°C for 60 min. Binding was terminated by aspirating the binding solution and washing the cells three times with 0.5 ml of ice-cold PBS. Cells were solubilized in 250 μl of lysis buffer (0.25 N NaOH, 0.05% SDS) and counts determined using a gamma-counter (Hewlett-Packard). The Bmax and Kd were calculated using Prism 3.03 software (GraphPad). Protein was determined using Bio-Rad’s DC protein assay kit.

Cell proliferation. Cell proliferation was determined using two methods: 1) the Wst-1 assay (Roche), which measures metabolic activity of the cells and, 2) by counting cells with a Coulter counter (Beckman). For the Wst-1 assay, transiently transfected cells were grown in serum-free medium or serum-free medium containing AT1 blockers [10^{-6} mol/l, losartan (Merck), valsartan (Novartis), candesartan (AstraZeneca), or telmisartan (Boehringer-Ingelheim), as described for each figure] or extracellular ANG II (eANG II, 1 × 10^{-7} mol/l) and cell proliferation measured 48 h thereafter. After the medium was removed, 100 μl per well of 10% Wst-1 (in serum-free medium) was added to the cells. The plates were prerared immediately on a plate reader at 450 nm to obtain a background count. After incubation at 37°C for 30 min, the plates were read again to determine the color density, which was proportional to the number of the cells. For stably transfected cells, the cells were seeded in 96-well plates at a density of 1.5 × 10^4 cells per well. The following day, medium was changed to serum-free medium and the Wst-1 assay performed on one set of cells to obtain time 0 values. Other sets of cells were incubated in fresh serum-free medium with or without drugs or eANG II (as described above) and the Wst-1 assay performed every 24 h, until 72 h. To determine cell proliferation by counting cell numbers, the stably transfected cells were seeded in growth medium (F12-K) containing 10% FBS in 24-well plates at a density of 6.25 × 10^4 cells per well. After 24 h, the medium was replaced with serum-free medium and one set of cells counted with a Coulter counter after trypsinization and resuspension in PBS. This represented the time 0 cell count. Similarly, for other sets of cells, medium was replaced with or without additives, and cells were counted every 24 h, up to 72 h.

Statistical analysis. Values are expressed as means ± SE. ANOVA with Tukey’s post hoc test was used for statistical analysis. P < 0.05 was considered statistically significant.

RESULTS

AT1 expression in CHO cells. Radioligand binding assay showed that CHO cells expressed very little ANG II receptor (16 ± 5 fmol/mg protein, Fig. 1B), while stably transfected CHO-AT1 cells showed high levels of AT1 as expected (5,399 ± 1,229 fmol/mg protein, Fig. 1A). The binding of the labeled ANG II to the cells was completely blocked by losartan, but not by the AT2 antagonist PD123319, suggesting that
these cells expressed only AT₁ receptor (Fig. 1, C and D). Similarly, the observation of low or undetectable levels of AT₁ receptors in CHO cells, which do not couple to ANG II-induced responses (inositol phosphate detection, thymidine incorporation, and cell division), has been reported by other investigators (30, 42).

**Intracellular ANG II expression in CHO and CHO-AT₁ cells.** Because CHO cells might not produce the necessary enzymes for processing of angiotensinogen to ANG II, the ANG II expression vector, pcDNA/TO-iANG II, contained sequence that coded for the mature ANG II peptide without a secretory signal. We performed experiments with both transiently and stably transfected cells. Production of ANG II in these cells was measured by a competitive ELISA in cell lysates, as well as in culture medium. Stable clones expressing similar levels of ANG II (CHO-iA, 119 ± 6; CHO-AT₁-iA, 91 ± 3 pg/mg protein) were chosen for the study. The iANG II concentration in stably transfected cells is similar to that reported in lysates of human hyperglycemic mesangial cells (25) and rat cardiac myocytes (our unpublished observations).

No ANG II was detected in naïve CHO cells or the control medium. As shown in Fig. 3A, CHO-iA expressed only AT₁ receptor (Fig. 1, A and B). This is consistent with the requirement of AT₁ for eANG II effects. However, iANG II resulted in cell proliferation in both CHO and CHO-AT₁ cells (Fig. 2. iANG II-induced cell proliferation was not statistically different than that induced by eANG II. To determine whether an AT₁ receptor blocker could inhibit the ANG II-induced cell proliferation, losartan (1 × 10⁻⁶ mol/l) was included in the culture medium. As shown in Fig. 2A, losartan completely blocked the effects of eANG II in CHO-AT₁ cells, but not the effects of iANG II in either cell type. These data suggest that CHO cells have 1) cellular machinery to induce intracrine effects and are a suitable model to study intracrine mechanisms, and 2) that AT₁ is not required for intracrine effects of ANG II.

**Stable transfection of CHO and CHO-AT₁ Cells for iANG II expression.** To further confirm the intracrine growth effects of iANG II, a growth curve of stable iANG II-expressing cells was determined after transferring the cells to serum-free medium. As shown in Fig. 3A, iANG II-expressing cells, CHO-iA (top) and CHO-AT₁-iA (bottom), proliferated at a significantly faster rate compared with the corresponding nonexpressing CHO and CHO-AT₁ cells, as determined by cell counts. These observations were confirmed by measuring cell proliferation using the Wst-1 assay (Fig. 3B, top and bottom).

**Effect of AT₁ receptor blockers on growth rate of stably transfected cells.** In stably transfected cells, as with transient expression, the AT₁ antagonist valsartan did not attenuate the faster growth rate of iANG II-expressing cells, but completely blocked the effect of eANG II (Fig. 4). In a separate experiment, we tested several AT₁ blockers, candesartan, losartan, telmisartan, and valsartan, to determine whether the different lipophilicities of these inhibitors would have any effect. None of the antagonists inhibited the intracrine effects of ANG II in either CHO-iA or CHO-AT₁-iA cells (Fig. 5).

**Additive effects of intra- and extracellular ANG II.** Because only extracellular and not intracellular effects of ANG II are blocked by AT₁ receptor antagonists, we hypothesized that together, extra- and intracellular ANG II would produce an additive effect. Indeed, eANG II produced further growth in CHO-AT₁-iA cells compared with CHO-iA cells. The additional growth effect induced by eANG II in CHO-AT₁-iA cells could be selectively blocked by AT₁ antagonists (Fig. 6).

**DISCUSSION**

In this report we demonstrate that AT₁ receptor is not required for the intracrine effects of ANG II. Intracrine effects of ANG II in cardiac myocytes and other cells have previously been reported (1, 5, 9, 11, 15, 16, 20, 46). It has been proposed that the intracrine effects of ANG II are mediated by an intracellular/nuclear AT₁ or AT₁-like receptor. However, AT₁ antagonists have shown mixed efficacy in blocking intracellular effects of ANG II. In hamster cardiac myocytes, gap junction conduction was blocked by intracellular losartan, but not the inward Ca²⁺ current in rat and hamster myocytes (10, 11). In the A7r5 smooth muscle cell line, cellular growth promoted by iANG II showed limited sensitivity to an AT₁ antagonist.
antagonist (17). In our earlier studies with cardiac myocytes, losartan did not block intracrine ANG II-induced cell growth. Neither did the antagonist inhibit cardiac hypertrophy in mice with cardiac-specific expression of iANG II. Thus the requirement for AT1 in intracrine effects of ANG II is not clear. Here, we determined if iANG II could act without AT1 receptors.

CHO cells have been extensively used to study the signaling mechanisms of specific AT1 receptor subtypes, because these cells express little or undetectable plasma membrane AT1 receptor (30, 42). Intracellular AT1 receptor was also not detected in the CHO cells (19). Expression of iANG II did not increase radioligand binding in CHO-iA cells, compared with CHO cells (data not shown), indicating that iANG II did not upregulate endogenous AT1 receptor. Consistent with that, eANG II did not elicit any biological effect in CHO cells, as shown above and by others (30, 42). However, when transfected with a plasmid that encodes for iANG II peptide, both CHO and CHO-AT1 cells showed significantly enhanced pro-

**Fig. 3.** Stable expression of iANG II in CHO and CHO-AT1 cells increases the proliferation rate, as determined by cell counts and Wst-1 assay. A: The stable iANG II-expressing cells, CHO-iA (top) and CHO-AT1-iA (bottom), and parent cells CHO (top) and CHO-AT1 (bottom), were seeded in 24-well dishes and transferred to serum-free medium after 24 h. Cell number was determined using a Coulter counter, at the time of placement in serum-free medium (time 0) and every 24 h thereafter, up to 72 h. B: Cells were seeded in 96-well dishes, transferred to serum-free medium after 24 h, and cell proliferation was measured by Wst-1 assay at time 0 and every 24 h thereafter, up to 72 h. Data are expressed as means ± SE; N = 6; *P < 0.05.

**Fig. 4.** The AT1 receptor blocker, valsartan, inhibits extracellular ANG II (eANG II), but not iANG II, induced cell proliferation. The experiment was performed as described in Fig. 3A. Valsartan (Val, 1 μmol/l) and/or eANG II (100 nmol/l) was added to the culture medium when cells were transferred to serum-free medium. The medium with the additives was replaced every 24 h. The results are expressed as relative cell numbers, after 72 h of Cont (non-iANG II-expressing cells), iANG II (iANG II-expressing cells), or eANG II (Cont in presence of 100 nmol/l ANG II). A: CHO cells. B: CHO-AT1 cells. Data are expressed as means ± SE; N = 6; *P < 0.05 vs. Cont.

**Fig. 5.** Effect of different AT1 receptor blockers on iANG II-induced cell proliferation. The experiment was performed as described in Fig. 3B. Angiotensin receptor blockers [candesartan (Candi), Los, telmisartan (Telmi), and Val, 1 μmol/l] were added to the culture medium when cells were transferred to serum-free medium. The medium with the additives was replaced every 24 h. The results are expressed as relative optical density, after 72 h in the serum-free medium. A: CHO and CHO-iA cells. B: CHO-AT1 and CHO-AT1-iA cells. Data are expressed as means ± SE; N = 6; *P < 0.05.
liferation. This plasmid contained the same expression cassette that we had used earlier in studies with cardiac myocytes (1). The iANG II concentration in the cells was comparable to that reported in human hyperglycemic mesangial cells (25), cardiac myocytes (our unpublished data), and in other studies with iANG II (7). The recombinant ANG II is retained inside the cells and is not detectable in the culture medium. Interestingly, iANG II-induced cell proliferation in CHO cells was not significantly different from that in CHO-AT1 cells, further suggesting no dependence of the intracrine mechanism on AT1.

This observation is in contrast to that of Cook et al. (8), who reported that iANG II increased mitogenic index in CHO cells only when iANG II is co-transfected with the AT1 receptor construct. The discrepancy between the two observations could be related to a number of explanations. Cook et al. performed transient transfection, followed by incubation of the cells in a medium containing 2% serum and then determined mitogenic index or cell numbers. The presence of serum may have masked the intracellular effects of ANG II. The second possibility lies in the fact that they expressed iANG II as a fusion protein with cyan fluorescent protein (ECFP), whereas our expression cassette produced native ANG II peptide, with only a single extra methionine at the NH2 terminus. Although, in their case, fusion of ANG II with ECFP did not prevent interaction with AT1 receptor and AT1-mediated effects, this could have affected possible interactions of ANG II with chromatin or other putative intracellular substrates. The use of serum, and the use of ANG II in a fusion protein might explain why they did not observe a mitogenic effect of iANG II, in the absence of AT1 receptors.

We did not observe an inhibitory effect of extracellular losartan on intracrine ANG II-induced cell proliferation, which was in agreement with other studies in cardiac myocytes (10, 15). AT1 antagonists differ in pharmacological properties, such as lipophilicity and surmountability, which might influence intracellular efficacy (29). Although there is no direct evidence for cellular internalization, intracellular efficacy of angiotensin receptor blockers (ARBs) was demonstrated by the effect on peroxisome proliferator-activated receptor-γ activation (37). The latter correlated with the degree of lipophilicity among ARBs and was not dependent on internalization via AT1 receptor (37). We tested four different AT1 antagonists to determine if more lipophilicity would block intracrine effects of iANG II. Even telmisartan, which is insurmountable and the most lipophilic (26), was not effective. We could not test higher concentrations of the ARBs because of a nonspecific effect on cell growth. However, Cook et al. (8) could inhibit intracrine effects of ANG II in CHO cells, when iANG II was co-transfected with AT1, using the same concentration of extracellular losartan. Again, the possible explanation lies in the fusion iANG II construct used in the study, which could possibly produce only AT1-mediated effects, as discussed above.

If iANG II action is independent of AT1 receptors, then why was losartan effective in inhibiting the intracrine effects in some studies (10)? An analysis of earlier studies with iANG II has suggested interaction with multiple intracellular receptors/proteins that couple to different intracrine effects (17). It is likely that involvement of AT1 receptors depends on the functional parameter studied, how ANG II originates inside the cells, and the subcellular location of agonist. Various techniques; microinjection, liposome-mediated delivery, and intracellular synthesis by a recombinant approach, have been used with iANG II (1, 9, 10, 16, 20). Our method of intracellular generation of minimally altered recombinant ANG II demonstrates that the AT1 receptor is not required for intracrine effects.

In accordance with our hypothesis for two different mechanisms, AT1-dependent and -independent, for hormonal and intracrine effects of ANG II, respectively, we observed an additive effect on cell proliferation when ANG II was present, both inside and outside of the cells. The AT1 antagonist valsartan selectively blocked only the extracellular effects. The signaling pathways activated by eANG II appear to be more potent than the intracellular mechanism, as evidenced by the greater cell proliferation induced by eANG II (Fig. 4). We did not see a significant difference in proliferation rate induced by iANG II in CHO and CHO-AT1 cells.

The nature of a possible AT1-independent intracrine mechanism for ANG II is not clear. A plausible explanation seems to be a direct effect of ANG II on chromatin and gene expression, as suggested by the work of Re et al. (33, 35). Intranuclear localization of ANG II after AT1-mediated internalization or intracellular synthesis, and direct binding to chromatin and an effect on gene expression (8, 13, 14, 33, 35), are all findings in support of this hypothesis. Insulin, PDGF, and FGF-2 also demonstrate an intracrine mode of action and can associate with chromatin, suggesting a direct modulation of chromatin structure and gene expression (22). FGF-2 was recently shown to directly interact with upstream binding factor, an architectural transcription factor in the nucleus, which regulates RNA transcription (39). Thus involvement of novel intracellular receptors or proteins in intracrine effects of ANG II represents an attractive hypothesis.

This is the first report to demonstrate ANG II activity in the absence of "known" receptors for the peptide. Together with other studies, these findings suggest that intracrine ANG II can act with and without AT1 receptor. ARBs have been reported...
to block hormonal, as well as AT1-dependent intracrine effects of ANG II (17). However, these drugs do not inhibit AT1-independent intracrine effects. The physiological significance of such effects is not directly evident from this study, but is suggested by several recent reports. Intracranial ANG II levels are increased in the diabetic human heart (18). High glucose increases intracranial renin activity in rat mesangial cells, and thus ANG II generation (45). In human mesangial cells high glucose increased intracranial ANG II, but not in the medium (25). In “The Losartan Intervention For Endpoint (LIFE) Reduction in Hypertension Study,” hypertensive patients with diabetes showed less regression of left ventricular hypertrophy than patients without diabetes, in response to antihypertensive therapy that included the AT1 antagonist losartan (31). These studies suggest a role for intracrine ANG II in diabetes, that may not be blocked by AT1 antagonists. Thus the intracrine system might be selectively activated in association with certain conditions and/or cell types (24, 40), thereby contributing to specific renin-angiotensin system compartments. Identification of intracrine ANG II mechanisms may lead to the development of molecular or pharmacological interventions that could be targeted to specific renin-angiotensin system compartments.

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REFERENCES


