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Intracellular Enhanced Cyan Fluorescent Protein/Angiotensin II Does Not Modify Angiotensinogen Accumulation in Transgenic Mice

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ABSTRACT

Background: Several studies suggest that extracellular angiotensin can upregulate renin and angiotensinogen (AGT). We have shown that enhanced cyan fluorescent protein/angiotensin II (ECFP/AngII) transgenic mice, in which AngII is fused downstream of ECFP and regulated by the mouse metallothionein housekeeping gene, possess elevated blood pressure and kidney thrombotic microangiopathy. The present study evaluated the effect of intracellular AngII on AGT messenger RNA (mRNA) and protein levels in ECFP/AngII transgenic mice.

Methods: The traditional guanidinium thiocyanate method was used to extract total mRNA. Proteins were extracted by homogenization in a tissue extraction reagent buffer. Northern blots for AGT mRNA and an 18S ribosomal RNA control were performed. Immunoblots for AGT protein levels with actin and tubulin controls were evaluated.

Results: Northern blot densitometry showed liver mRNA levels an average of 12-fold greater than levels in the brain or kidney in both Lines A and D (different copies of the transgene) with no quantifiable differences between wild-type (WT) and homozygous (HO) transgenic mice. Immunoblots showed liver AGT protein levels 3.2-fold greater than levels in the brain or kidney, with no differences observed between WT and HO transgenic mice.

Conclusion: ECFP/AngII transgene expression does not alter AGT mRNA or protein levels in major organs (kidney, liver, and brain) of transgenic mice. The altered blood pressure and kidney thrombosis observed in these transgenic mouse lines are not the result of increased intracellular AGT synthesis and resultant increases in free extracellular AngII. This finding is consistent with our published studies that indicate no increase in circulating AngII by radioimmunoassay.

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INTRODUCTION

Since the discovery of the renin-angiotensin system (RAS) more than a century ago, investigations have shown that components of the RAS possess functionality far beyond the effects of circulating angiotensin II (AngII) on blood pressure and fluid balance. AngII, the end product of the RAS cascade, has been implicated in the disease pathogenesis pathways[1,2] leading to cardiovascular and renal disorders. More recently, the intracellular or intracrine effects of members of the RAS, particularly with regard to cardiovascular and renal pathologies, have increasingly come under study. Multiple intracrine signaling pathways[3,4] have been proposed, including those that involve intracellular organelles and compartments, such as mitochondria, the endoplasmic reticulum, the Golgi, and the nucleus, among others.[5]

Many of our published reports over the past decade have focused on the existence and function of intracrine AngII.[6] Moreover, we have shown that a fluorescent fusion consisting of AngII fused downstream and in frame with enhanced cyan fluorescent protein (ECFP/AngII), when genetically expressed in cells, contributes to cell proliferation, cyclic adenosine monophosphate response element-binding protein stimulation, and redistribution of the AngII receptor AT_1 (AT_1R).[7] This construct encodes a form of AngII

that lacks a secretory signal and, hence, is retained within cells.[8] To further study the in vivo effects of intracellular AngII, we developed transgenic mice that express ECFP/AngII regulated by the metallothionein promoter and found that these mice demonstrate elevated blood pressure (as measured by radiotelemetry) and renal thrombotic microangiopathy.[8] In light of these findings, we sought to determine the mechanism by which intracellular AngII (which acts through a mechanism distinct from canonical G protein–coupled cell surface receptor signaling) might mediate kidney and blood pressure activities.

The major components of the RAS, including angiotensinogen (AGT) and AngII have been found to be synthesized in most tissues. Schunkert et al[9] showed that augmented extracellular AngII, generated through plasma infusion of AngII, increases AGT messenger RNA (mRNA) levels in rat kidneys and suppresses renin levels. However, 3 days of angiotensin-converting enzyme inhibition did not alter AGT mRNA or AngII in the kidney and liver.[9] In cultured cardiomyocytes, Tamura et al[10] found increased AGT mRNA due to mechanical stretch-induced AngII. They also investigated the effects of AT_1R blockade and RNA synthesis inhibition, both of which reduced AGT.

High glucose upregulates production of intracellular AngII.[11] Some renin inhibitors inhibit intracellular accumulation of AngII,[12] reversing the effects of hyperglycemia on organs.

Based on our prior findings and the collective reports described above, we sought to determine whether intracellular AngII (as ECFP/AngII) similarly regulated AGT accumulation.

METHODS

We compared mRNA and protein levels in the liver, kidney, and brain using Northern blotting and immunoblotting for both Line A (15 copies of the transgene) and Line D (150 copies of the transgene) of our ECFP/AngII transgenic mice. Entire organs were either flash frozen or processed fresh after removal.

Northern Blots

The organs (kidney, liver, and brain) were harvested from mice, and mRNA was extracted using the traditional guanidinium thiocyanate method[13] using the entire organ. Total mRNA was further purified using the RNeasy kit (QIAGEN, Valencia, CA). Ten μg of mRNA per sample underwent electrophoresis on a 1% agarose, 2.2 M formaldehyde gel. Samples were thereafter transferred overnight to zeta probe membranes (BIO-RAD, Hercules, CA). Membranes were hybridized using rat AGT cDNA and an 18S ribosomal RNA fragment to control for differential loading of samples, radiolabeled with αP-32 deoxycytidine triphosphate (Perkin Elmer, Waltham, MA) and the Prime-It labeling kit (Agilent Technologies, Santa Clara, CA). The results were quantified by densitometry.

Immunoblots

The organs were flash frozen after harvest. Protein was collected using Tissue Extraction Reagent I lysis buffer (Life Technologies, Carlsbad, CA) at 400 μL per 0.1 g of tissue. For each sample, 100 μg was electrophoresed on a 4%-12% Bis-Tris acrylamide gradient gel (Life Technologies). Primary antibodies used were anti-AGT (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-beta actin (Cell Signaling Technology, Beverly, MA). Secondary antibodies were horseradish peroxidase (HRP) anti-goat (abcam, Cambridge, MA) and HRP anti-rabbit (Amersham, GE Healthcare, Little Chalfont, UK).

Statistical Analyses

Statistical *P* values were calculated using analysis of variance with the Bonferroni post hoc test.

RESULTS

The Northern blots were consistent with a 12-fold increase in liver AGT mRNA levels compared to the kidney or brain (Figure 1). We observed no significant difference between wild-type (WT) and homozygous (HO) littermates. Immunoblots showed a 3.2-fold increase in AGT levels in the liver compared to the kidney or brain but no significant difference between levels in WT and HO mice (Figure 2).

DISCUSSION

Extracellular AngII has been shown to upregulate AGT in a positive feedback loop in some systems. Sechi and colleagues[14] did not observe a significant difference in renal or hepatic AGT mRNA levels following 7 days of AngII infusion at 200 ng/kg/min in rats. Similarly, O'Callaghan et al[15] found no difference in AGT expression following AngII exposure in cultured astrocytes derived from neonatal C57BL6 mice. However, Eggena et al[16] found AngII to upregulate AGT mRNA transcription in isolated nuclei from rat hepatic cells in a dose-dependent fashion that progressively declined with higher doses of AngII. Similarly, Klett and colleagues[17] reported AngII-mediated (concentrations of 9 and 90 nmol AngII) upregulation of AGT mRNA in freshly isolated rat hepatocytes. Kobori et al[18] observed significant increases in AGT mRNA and protein (glycosylated form) expression in kidney and liver following AngII-induced hypertension in rats infused with AngII (80 ng/kg/min) for 13 days. Kobori et al[19] also showed

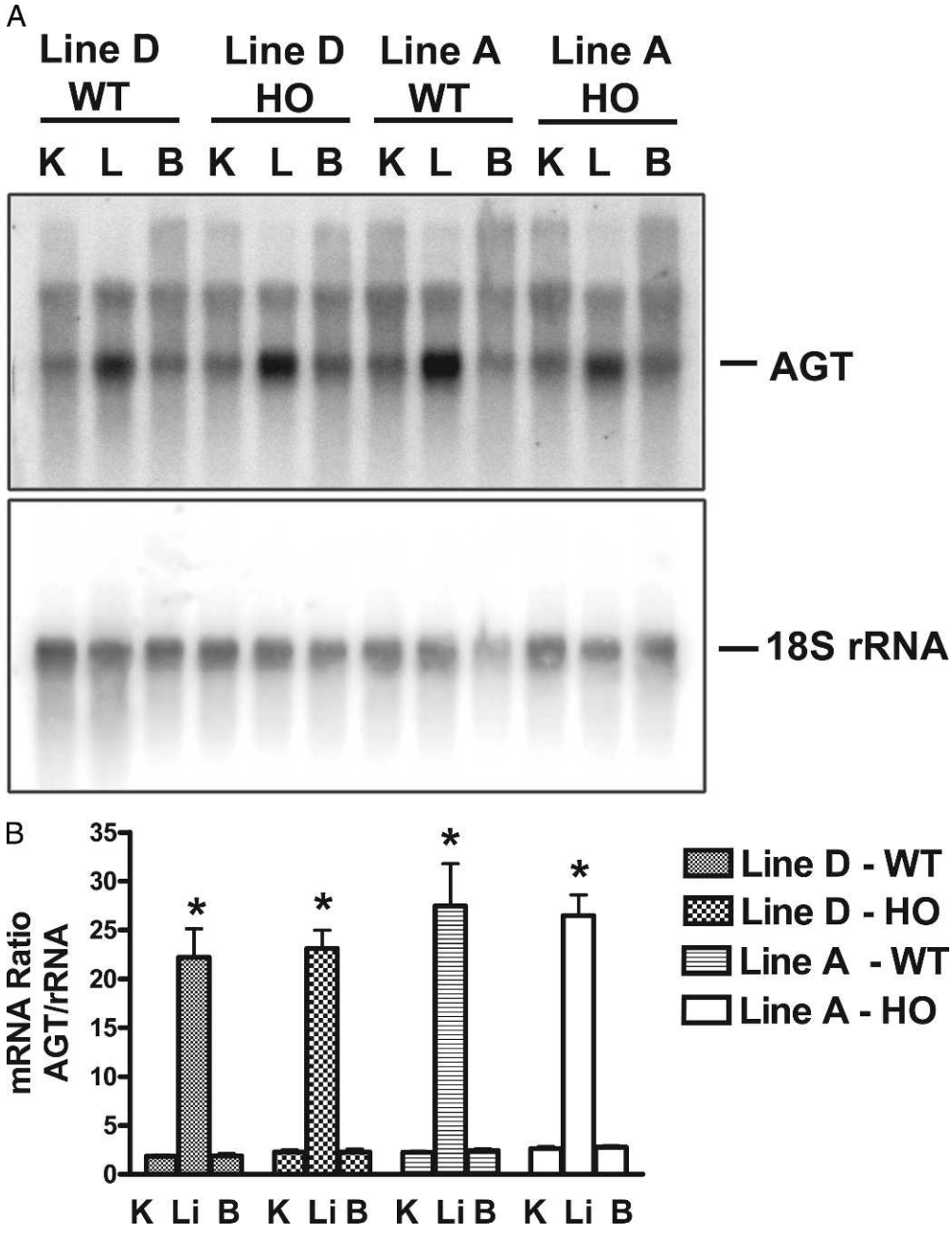

Figure 1. Angiotensinogen (AGT) messenger RNA (mRNA) levels in the kidney, liver, and brain are not significantly different between homozygous (HO) and wild-type (WT) mice. (1A) Northern blot was performed with total mRNA extracted from the kidney (K), liver (L), and brain (B) of WT and transgenic HO mice to evaluate AGT expression. Filters were rescreened with an 18S ribosomal RNA fragment control to standardize mRNA loading and transfer between different samples. (1B) Densitometry shows that liver (Li) mRNA levels are an average of 12-fold greater (*$P<0.001$) than brain (B) or kidney (K) levels in both Lines A and D with no quantifiable differences between WT and HO transgenic mice.

significantly increased AGT mRNA and protein levels in the kidney, liver, and plasma of rats fed a high-salt diet and infused with AngII (40 ng/kg/min).

In our experiments, we observed no changes in AGT mRNA and protein levels in ECFP/AngII transgenic mice as compared to WT mice in the organs investigated. The RAS intracrine pathways are diverse and involve many mechanisms and intracellular compartments.[2,20] The intracellular functions of AngII are also diverse, and reported results are cell-type

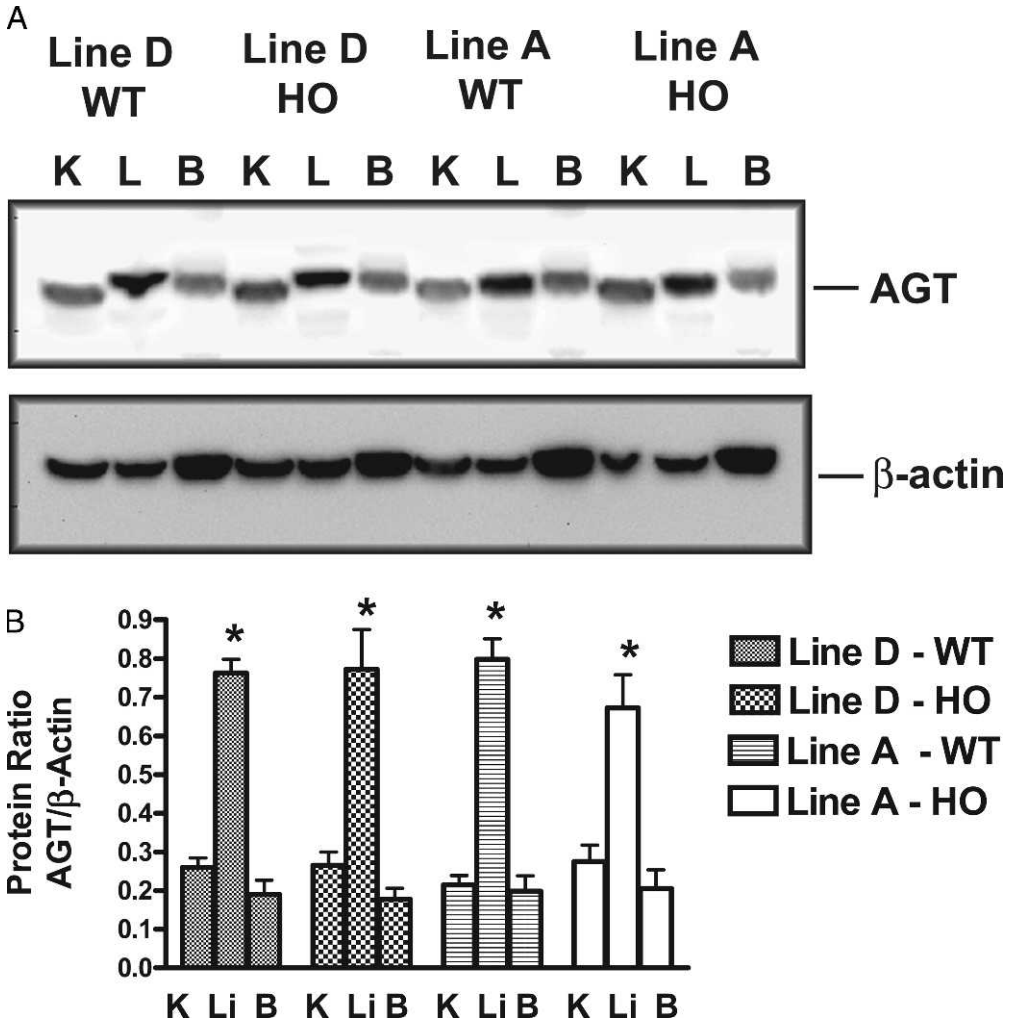

Figure 2. Angiotensinogen (AGT) protein levels in the kidney, liver, and brain of homozygous (HO) transgenic versus wild-type (WT) mice are not significantly different. (2A) Proteins were extracted from kidney (K), liver (L), and brain (B) of HO and WT mice, and immunoblots were performed. Immunoblots were rescreened with β-actin control to standardize for variations in protein loading and transfer. (2B) Liver (Li) AGT protein levels are 3.2-fold greater (*$P<0.001$) than brain (B) or kidney (K) levels with no observed difference between WT and HO transgenic mice.

and model dependent.[14] ECFP/AngII possesses no signal peptide and is designed to be retained within the cell of synthesis. To date, all data suggest that ECFP/AngII is not released through conventional or nonconventional means. In earlier published studies, we reported no difference in plasma AngII levels of HO and WT mice by radioimmunoassay.[8] Observations by Tamura et al[10] suggest that AngII activates AGT transcription only through the classic AT_1R pathway in cardiac myocytes. Therefore, the collective published data[9,10,16-19] suggest that AngII activation of plasma membrane-associated G protein–coupled receptor with concomitant G-protein stimulation and subsequent downstream signaling leads to AGT accumulation.

CONCLUSION

Intracellular AngII, acting through noncanonical pathways, does not necessarily stimulate AGT production and accumulation. Moreover, blood pressure elevation may be independent of the localization of AngII. Intracellular AngII may increase blood pressure through mechanisms distinct from extracellular AngII.

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