AT_1 receptor blockade delays postlactational mammary gland involution: a novel role for the renin angiotensin system

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ABSTRACT **Angiotensin II (AngII), the main effector peptide of the renin-angiotensin system (RAS), participates in multiple biological processes, including cell growth, apoptosis, and tissue remodeling. Since AngII activates, in different cell types, signal transducing pathways that are critical for mammary gland postlactational regression, we investigated the role of the RAS during this process. We found that exogenous administration of AngII in mammary glands of lactating Balb/c mice induced epithelium apoptosis [2.9±0.5% (control) *vs.* 9.6±1.1% (AngII); $P < 0.001$] and activation of the proapoptotic factor STAT3, an effect inhibited by irbesartan, an AT_1 receptor blocker. Subsequently, we studied the expression kinetics of RAS components during involution. We found that angiotensin-converting enzyme (ACE) mRNA expression peaked 6 h after weaning (5.7-fold; $P<0.01$), while induction of angiotensinogen and AT_1 and AT_2 receptors expression was detected 96 h after weaning (6.2-, 10-, and 6.2-fold increase, respectively; $P<0.01$). To assess the role of endogenously generated AngII, mice were treated with losartan, an AT_1 receptor blocker, during mammary involution. Mammary glands from losartan-treated mice showed activation of the survival factors AKT and BCL_{-XL}, significantly lower LIF and TNF-α mRNA expression ($P<0.05$), reduced apoptosis [12.1±2.1% (control) *vs.* 4.8±0.7% (losartan); $P<0.001$] and shedding of epithelial cells, inhibition of MMP-9 activity in a dose-dependent manner (80%; $P<0.05$; with losartan IC_{50} value of 6.9 mg/kg/d] and lower collagen deposition and adipocyte invasion causing a delayed involution compared to vehicle-treated mice. Furthermore, mammary glands of forced weaned AT_{1A}- and/or AT_{1B}-deficient mice exhibited retarded apoptosis of epithelial cells [6.3±0.95% (WT) *vs.* 3.3±0.56% (AT_{1A}/AT_{1B} DKO); $P<0.05$] with remarkable delayed postlactational regression compared to wild-type animals. Taken together, these results strongly suggest that AngII, *via* the AT_1 receptor, plays a major role in mouse mammary gland involution identifying a novel role for the RAS.—Nahmod, K. A., Walther, T., Cambados, N., Fernandez, N., Meiss, R., Tappenbeck, N., Wang, Y., Raffo, D., Simian, M., Schwiebs, A., Pozner, R. G., Fuxman Bass, J. I., Pozzi, A. G., Geffner, J. R., Kordon, E. C., Schere-Levy, C. AT_1 receptor blockade delays postlactational mammary gland involution: a novel role for the renin angiotensin system. *FASEB J.* 26, 1982–1994 (2012). www.fasebj.org**

Key Words: AKT · angiotensin II · apoptosis · metalloproteases · STAT3

THE RENIN-ANGIOTENSIN SYSTEM (RAS) was originally described as an endocrine system in which the substrate protein angiotensinogen (AGT) is sequentially cleaved by peptidases, renin, and angiotensin-converting en-

Abbreviations: ACE, angiotensin converting enzyme; AGT, angiotensinogen; Agtr1a, angiotensin II receptor subtype 1A gene; Agtr1b, angiotensin II receptor subtype 1B gene; AngII, angiotensin II; AT_1, angiotensin II type 1 receptor; AT_{1A}, angiotensin II subtype 1A receptor; AT_{1A}KO, angiotensin II subtype 1A receptor knockout; AT_{1B}, angiotensin II subtype 1B receptor; AT_{1B}KO, angiotensin II subtype 1B receptor knockout; AT_2, angiotensin II type 2 receptor; DKO, double knockout; ECM, extracellular matrix; IGF-1, insulin-like growth factor-1; JAK, Janus kinase; LIF, leukemia inhibitory factor; Los, losartan; MMP, matrix metalloproteases; PRL, prolactin; RAS, renin-angiotensin system; RPA, RNase protection assay; STAT, signal and activator of transduction; WT, wild-type.

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doi: 10.1096/fj.11-191932

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zyme (ACE), to form the biologically active octapeptide angiotensin II (AngII). A large body of evidence supports the existence of numerous organ- or tissue-based RASs where AngII is locally produced, exerting diverse physiological and pathophysiological effects (1–4). In addition to its role in homeostasis of cardiovascular and renal systems, AngII exerts various actions in a paracrine and autocrine manner, influencing cell growth, differentiation, and apoptosis, and is considered a true cytokine (5–7).

AngII acts through two different G-protein-coupled receptors, angiotensin II type 1 receptor (AT_1) and angiotensin II type 2 receptor (AT_2), which have distinctive pharmacological and signal-transducing characteristics (8). Rodents carry two isoforms of the AT_1 receptor, AT_{1A} and AT_{1B}, that are products of differentially expressed and regulated genes (9). Most of the known actions of AngII are mediated by the AT_1, which represents a critical pharmacological target in the treatment of cardiovascular disorders (10).

The mammary gland undergoes repeated cycles of growth, differentiation, and regression. During pregnancy, the secretory alveolar lobules develop on the structure of branching ducts until they completely penetrate the stromal tissue. The lobuloalveolar structures persist until the end of lactation. During lactation, terminal differentiation and milk production are the most important features. During this period, alveolar cell survival is maintained by suckling and signal transduction pathways, including prolactin (PRL)–signal and activator of transduction (STAT)5 and insulin-like growth factor-1 (IGF-1)–PI3K-AKT (11). Postlactational regression following weaning is divided in two distinct phases. In the early phase, which comprises the first 48 h after weaning, milk stasis results in the induction of local factors that lead to massive apoptosis of the alveolar epithelium. Programmed cell death is initiated within 12 h of pup removal and involves activation of STAT3 and caspase-3 cleavage (12). Leukemia inhibitory factor (LIF) appears to be a key activator of STAT3 (13, 14). The second stage of involution involves extensive tissue remodeling with spatial and temporal expression pattern of matrix metalloproteases (MMPs) that break down the extracellular matrix (ECM) surrounding the alveoli, leading to anoikis-induced apoptosis and collapse of the alveoli (15). Many other apoptotic pathways have been implicated during involution as well. These include members of the death receptor ligand family (DR)-like NF-κB/IκB kinase (IKK), TWEAK, TNF-α or Fas/Fas-L, and members of the Bcl-2 family. Deletion of the antiapoptotic BCL-X_L gene accelerates apoptosis, while loss of the proapoptotic Bax protein delays involution (16, 17). The expression of a constitutively active *Akt* transgene provides an overriding survival signal during involution, suggesting that pAKT could be a molecular sentinel for survival/death signals in mammary epithelium (18).

Recently, it has been shown that RAS components are expressed in epithelial ducts of normal and transformed mammary glands (19, 20). Furthermore, it has been reported that RAS is likely to be important in various steps of breast cancer progression (21, 22). Considering that AngII is known to activate, in different cell types and tissues, the signal transducing pathways that are critical during involution (23–28), we decided to study the contribution of the RAS to this process. Using two different approaches, pharmacological blockade of the AT_1 receptor and mice deficient in either the AT_{1A} or AT_{1B} or both receptors [double-knockout (DKO) mice], we studied the role of AT_1 in postlactational regression. Here, we report that AT_1 receptor signaling contributes with other local factors to induce apoptosis and tissue remodeling during mammary gland involution, identifying a novel role for the RAS.

MATERIALS AND METHODS

Cell culture and treatments

HC11 cells, derived from BALB/c mouse normal mammary glands (kindly provided by Dr. Nancy Hynes, Friedrich Meischer Institute, Basel, Switzerland), were cultured as described previously (29). After cells reached confluence, they were maintained in serum-free medium for 4 h for starvation before they were treated with AngII (10^{-6} M; Bachem AG, Bubendorf, Switzerland) or vehicle. Proteins were then extracted at different time points (5, 15, 30, 60, and 120 min).

Animals and experimental design

Wild-type Balb/c mice were obtained from the animal facilities of the Institute of Hematologic Research (National Academy of Medicine, Buenos Aires, Argentina). Mice lacking AT_{1A} or AT_{1B} receptors were generated using homologous recombination in embryonic stem cells, as described previously; AT_{1A} (−/−//+/+) and AT_{1B} (+/+//−/−) single-knockout mice were used to generate the AT_{1A}/AT_{1B} DKO mice (−/−//−/−) (30). To generate homozygous wild-type (WT; +/+//+/+) and DKO animals, heterozygous offspring were intercrossed and bred on a 129 × C57BL/6 background at the animal facilities of the University of Hull (Hull, UK), whereby experimental homozygous animals were generated by cross-breeding of heterozygous mice to ensure the most similar genetic background. Sets of virgin females at 3–6 mo of age were used for the experimental breeding.

All experiments were performed in accordance with the APS Guiding Principles for the Care and Use of Animals in Research (American Physiological Society) and the guidelines of federal law on the use of experimental animals in the UK and Argentina (Animal Welfare Act) and were approved by the local authorities.

All animals were given sterilized laboratory chow and water *ad libitum* and housed after parturition in solitary caging (with litter) under a standard 12-h light-dark photoperiod. Litters were normalized to 4–6 pups at parturition. Pups were removed from mice at d 8 of lactation. Tissue was aseptically removed from mice at lactation phase and 24, 48, 72, 96, or 120 h after forced weaning. The fourth inguinal pair of mammary glands was used for histological sectioning, RNA isolation, and protein isolation.

For *in vivo* experiments, lactating mice receiving local treatment were administered subcutaneous intramammary

injections with 100 μl AngII (10^{-6} M) solubilized in 0.9% NaCl, 0.9% NaCl alone (control), or irbesartan (10^{-5} M) + AngII (10^{-6} M) in a final volume of 100 μl using the same solvent. In all cases, AngII or solvent alone was injected into the contralateral fourth mammary gland as a control. For mice receiving systemic treatment with AngII, 150 μl of 10^{-5} M AngII diluted in 0.9% NaCl was administered subcutaneously in the upper back. Mice were sacrificed by cervical dislocation 30 min, 60 min, or 8 h later, and mammary glands were dissected. Mice treated systemically with losartan (Los; DuPont, Boston, MA, USA), an AT_1 receptor blocker, were injected with a daily dose of 20 mg/kg diluted in 0.9% NaCl or 0.9% NaCl alone subcutaneously in the upper back, as described previously by other authors (31). First dose was administered 1 d before forced weaning, while subsequent doses were administered once daily during involution. Mice were sacrificed by cervical dislocation at 24, 48, 72, 96, and 120 h after forced weaning, and mammary glands were removed for further analysis.

Polymerase chain reaction (PCR) for genotyping

Homozygosity in the 3 knockout genotypes used (AT_{1A}KO, AT_{1B}KO, and AT_{1A}/AT_{1B} DKO) has been proven by PCR after weaning and again after experiments were performed using specific primer pairs for each gene locus. Specific pairs of primers to detect the AT_{1A} and AT_{1B} receptor subtypes were described previously (32).

RNA isolation

Total RNA was extracted from mammary glands with SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Conventional and quantitative RT-PCR

Conventional and quantitative real-time PCR was performed as described previously (33).

Histology, immunohistochemistry, and TUNEL assay

For histological, immunohistochemical, and apoptosis analysis, mammary glands were immersed overnight in 10% buffered formalin and then embedded in paraffin. Morphological features were evaluated in hematoxylin-and-eosin (H&E)-stained slides. For ECM evaluation, Masson's Trichrome stain was used, where connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. For immunohistochemical assay, mammary tissue was deparaffinized and then incubated 10 min in 10 mM sodium citrate buffer (pH 6.0) at a subboiling temperature. Endogenous peroxidase activity and nonspecific binding were blocked by incubation in 5% hydrogen peroxide for 5 min and in 1% BSA in phosphate buffer (50 mM), 0.9% ClNa, and 0.1% Triton X-100 for 1 h at room temperature, respectively. Sections were incubated 24 h at 4°C with the following primary antibodies: anti-cleaved caspase-3 (catalog no. 9661; Cell Signaling Technology, Beverly, MA, USA) or anti-mouse STAT3 antibody (Clon C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1;100 as described previously (14). Immunoreactivity was detected by exposure to 3,3′-diaminobenzidine (DAB; Dako Cytomation, Carpinteria, CA, USA). Sections were counterstained with hematoxylin and mounted for conventional light microscopy. The specificity of the immunostaining was tested by omission of the primary antiserum. Controls gave negligible background staining (data not shown). Apoptotic cells were identified by TUNEL assay using the TdT-FragEL apoptosis detection system (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA) following the manufacturer's instructions. Apoptosis was evaluated by counting ≥1200 cells split among ≥10 randomly chosen fields at ×400 view. The percentage of apoptotic cells was calculated for each section, and the results are expressed as means ± SE.

Western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously (14).

Zymography

Mammary glands were homogenized on ice in RIPA protein extraction buffer as described above. The homogenates were centrifuged for 15 min at 4°C, and the supernatants were subjected to electrophoresis on gelatin substrate gels (8.8% SDS-polyacrylamide slab gels containing 1 mg/ml gelatin), as described previously (15). Subsequently, the gels were treated with 2.5% Triton X-100 for 30 min, followed by incubation for 24 h at 37°C in a buffer containing 100 mM Tris-HCl (pH 7.4) and 15 mM $CaCl_2$. Gels were stained with Coomassie blue R-250 and destained with water until clear zones indicative of proteolytic activity emerged against a blue background. The zymograms were scanned and subjected to densitometric analyses using the PC version of NIH image (Scion Corp., Frederick, MD, USA).

To determine IC_{50} values, MMP-9 activity kinetics at 96-h involution were carried out in the presence of variable daily doses of the AT_1 receptor blocker Los. IC_{50} values and corresponding maximal and minimal effects from dose-response curves were calculated by nonlineal regression analysis with best-fit values by GraphPad Prism (GraphPad, San Diego, CA, USA), assuming that the dose-response curve has a standard slope, equal to a Hill slope of −1. The responses are expressed as a percentage of the maximal MMP-9 activity at 96 h involution in the absence of the inhibitor Los, while minimal MMP-9 activity was considered the one measured during lactation. Experiments were run in triplicate.

RNase protection assay (RPA)

AT_{1A} expression was analyzed by RNase protection assays using the commercially available RPA II kit [Ambion (Europe) Ltd., Huntingdon, UK], according to the manufacturer's protocol. Total RNA (30 μg) was hybridized with one of the following probes: *MMAT1A*, a recently described vector (34) was used to transcribe a radioactive probe complementary to a 352-bp fragment specific for AT_{1A} mRNA; *rL32*, a commercially available rL32 probe template (BD PharMingen, San Diego, CA, USA), complementary to a 127-bp fragment of rL32 mRNA, was used as a housekeeping control, as described earlier (35). RNA samples were hybridized with 40,000 cpm of the radiolabeled probe for MMAT1A and 40,000 cpm of the radiolabeled probe for *rL32* as the housekeeping gene. The hybridized fragments protected from RNase A + T1 digestion were separated by electrophoresis on a denaturing gel (5% polyacrylamide, 8 M urea) and analyzed using a Fujix BAS 2000 PhosphoImager system (Raytest, Straubenhardt, Germany). Quantitative analysis was performed by measuring the intensity of the AT_{1A} bands normalized by the intensity of *rL32*.

Immunofluorescence

HC11 cells were grown on sterile chamber glass, fixed 20 min with 1% paraformaldehyde in PBS, washed twice with 1:10 PBS, incubated 15 min with 1:10 PBS and 0.1% Triton solution, and then blocked 1 h with 1:10 PBS, 0.025% Triton, and 5% BSA. Cells were then washed and incubated with the appropriate primary antibody: anti-AT_1 rabbit polyclonal (N10; sc1173), anti-AT_2 rabbit polyclonal (H-143; sc 9040) and anti-AGT goat polyclonal (N10; sc7419), or IgG rabbit polyclonal or IgG goat polyclonal as negative controls (all from Santa Cruz Biotechnology), diluted 1:50 in a 1:10 PBS, 0.025% Triton, and 5% BSA solution. After washing, cells were incubated with secondary antibody: Dylight 488-conjugated goat anti-rabbit or FITC-conjugated rabbit anti-goat (both from Jackson Immunoresearch, Baltimore, MD, USA) diluted 1:100 in 1:10 PBS, 0.025% Triton, and 5% BSA solution. After 5 washes with 1:10 PBS, phalloidin-TRITC (Sigma Aldrich, St. Louis, MO, USA) was added for 3 min. Sections were air-dried and mounted with Fluoromount for a subsequent analysis under an Olympus laser confocal microscope (Olympus, Tokyo, Japan).

Frozen mammary gland samples were cut using a cryostat to obtain 10- to 12-μm-thick sections, air-dried, and fixed for 20 min in 10% formalin in PBS. Sections were blocked with PBS and 2% FCS for 1 h and then treated with primary antibodies diluted in blocking buffer at a 1:100 dilution overnight at 4°C. After 3 washes in PBS, sections were incubated with fluorescein-conjugated secondary antibodies (1:100) for 1 h at room temperature in blocking buffer. Slides were washed with PBS, nuclei were stained with either propidium iodide or 4′, 6-diamino-2-phenylindole (Sigma), and slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Sections were analyzed under a Nikon laser confocal microscope (Nikon, Tokyo, Japan).

Statistical analysis

Statistical significance of differences was determined with Student's *t* test or by analysis of variance followed by Tukey *post hoc* test. A value of $P < 0.05$ was considered statistically significant. IC_{50} values and corresponding maximal effects from dose-response curves were calculated by nonlinear regression analysis by GraphPad Prism.

RESULTS

AngII signaling in mammary epithelial cells *in vitro*

The ability of AngII to trigger MAPK, AKT, NF-κB, and Janus kinase (JAK)/STAT3 activation has been described in several cell types and tissues (23, 24, 26, 27). To investigate signaling pathways that may be modulated by RAS in the mammary epithelium, we used the HC11 mouse mammary cell line. In a first set of experiments, we studied the expression of AT_1 and AT_2 receptors. We found that HC11 cells express AT_1 and AT_2 at the mRNA (**Fig. 1*A***) and protein level (Fig. 1*B*). Positive staining of AT_1, AT_2, and AGT were also demonstrated in mammary epithelial cells by confocal microscopy (Fig. 1*C*). As depicted in Fig. 1*B, C*, AT_1 expression is higher than that of AT_2. In a second set of experiments, we tested AngII for its ability to trigger phosphorylation of signaling proteins that are activated during mammary involution like MAPKs, NF-κB, and STAT3. We found that ERK1/2 and STAT3 phosphorylation peaked 5 min after addition of AngII, while

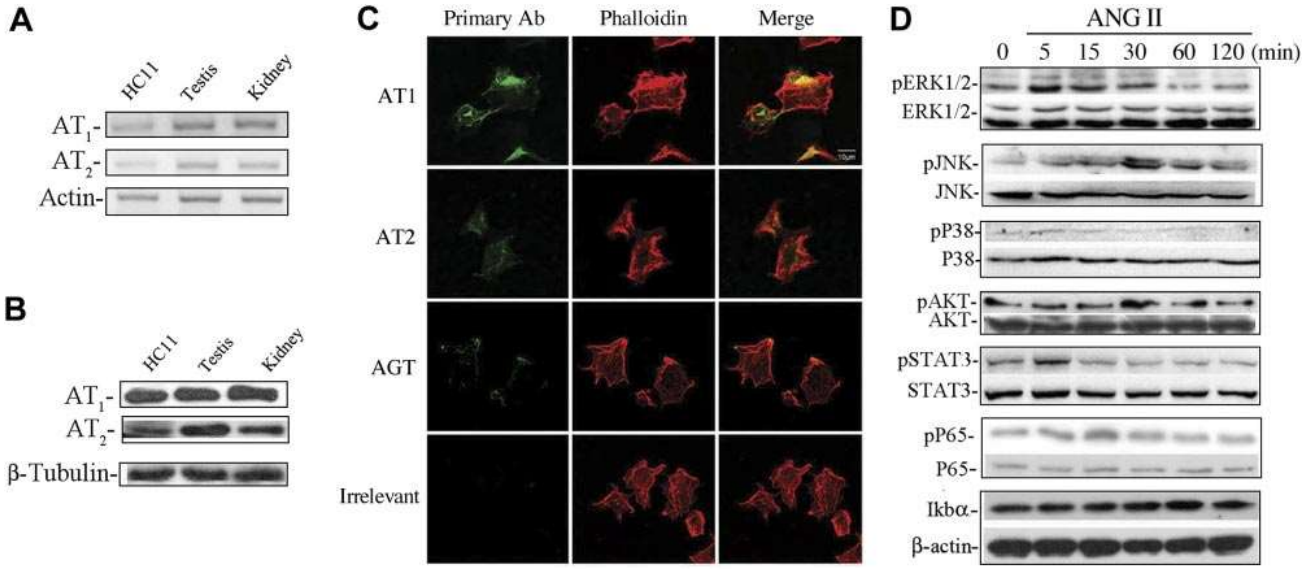

Figure 1. *A, B*) Expression of AT_1 and AT_2 in the murine mammary epithelial cell line HC11 as determined by RT-PCR (*A*) and Western blot (WB) analysis (*B*). The blot was probed with antibodies specific for AT_1, AT_2, and β-tubulin. Kidney and testicle tissues were used as positive controls for the expression of both receptors. *C*) Fixed cells were incubated with a primary polyclonal antibody against AT_1 (top panel), AT_2 (top middle panel), or AGT (bottom middle panel) and subsequently with a fluorochrome-conjugated secondary antibody (green) and analyzed by confocal microscopy (red, phalloidin; yellow, merge). Scale bar = 10 μm. *D*) HC11 cells were treated with AngII (10^{-6} M) for the indicated time, and WB analyses were performed using phospho-specific antibodies against ERK1/2, JNK, p38, AKT, STAT3, P65, and IKBα (top row for each kinase). Same samples were blotted with antibodies to account for total kinase expression (bottom row for each kinase) or β-actin. Results show 1 representative of ≥3 independent experiments.

pJNK reached a maximum 15 min after treatment. Phosphorylation of AKT and P65 was found at 30 and 15 min, respectively, while AngII stimulation did not induce significant changes in the phosphorylation levels of P38 or IκBα (Fig. 1*D*).

Exogenous administration of AngII in lactating mouse mammary glands induces involution-associated events

The ability of AngII to trigger signal transduction pathways in HC11 cells *in vitro* that are activated during involution raised the possibility that AngII may also activate these pathways in the mammary gland *in vivo*. The first stage of mammary gland involution is characterized by apoptosis of secretory cells. In particular, a key role has been well demonstrated for activated STAT3 in the induction of epithelial apoptosis during involution (12). Lactation represents an ideal phase to study proapoptotic signaling cascades in the mammary gland, since STAT3 remains dephosphorylated, in an inactive state in the cytoplasm, and the apoptosis rate of epithelial cells is very low. During involution, STAT3 is activated and translocated to the nucleus. To determine whether elevated AngII levels could induce involution-associated events, AngII was injected systemically (**Fig. 2*A***) or locally into the gland (Fig. 2*B*) of lactating females. To exclude the possibility that tissue distension secondary to injection in the mammary gland may trigger STAT3 activation, solvent alone was inoculated into the contralateral gland as a control. The Western blot analyses show that AngII strongly induced STAT3 phosphorylation when inoculated both systemically or into the gland. Figure 2*C* shows that AngII also induced ERK1/2 phosphorylation at 30 min. The coinjection of irbesartan, an AT_1 receptor blocker, in the mammary gland of lactating females partially blunted the AngII effects, implying that STAT3 (Fig. 2*D*) and ERK (Fig. 2*E*) activation is AT_1 mediated. We then examined STAT3 localization by immunohistochemical staining in mammary glands locally injected with AngII or vehicle. Significant increase in positive nuclear staining was observed in AngII-treated glands in comparison to vehicle-treated glands (Fig. 2*F*), illustrating the activation of STAT3 by AngII. As AngII induced STAT3 phosphorylation and translocation to the nucleus in epithelial cells of lactating mammary glands, we next investigated whether it could also trigger programmed cell death in this experimental setting. More than 3

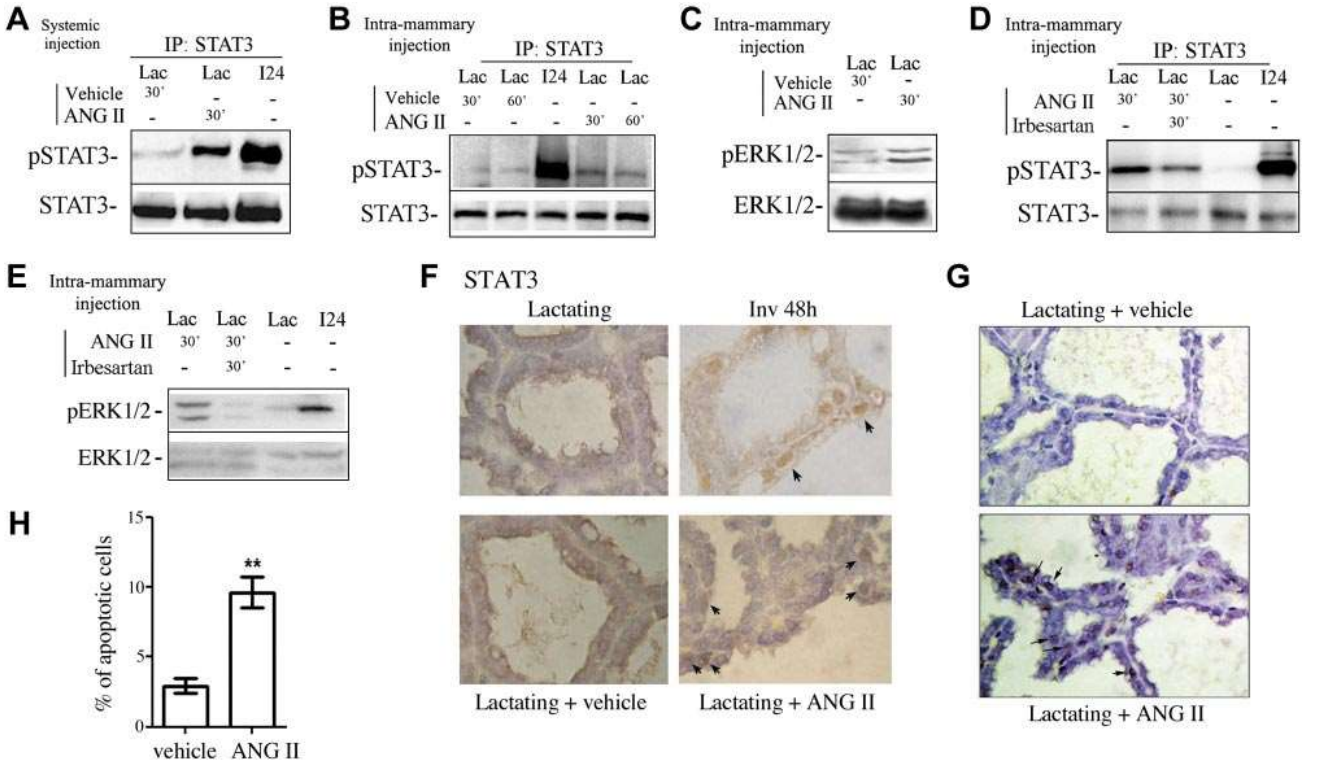

Figure 2. AngII induces STAT3 activation and cell death in epithelial cells of lactating mouse mammary glands. *A–E*) Lactating Balb/c mice were injected subcutaneously with AngII or vehicle in the upper back (*A*) or intramammary (*B*, *C*) or intramammary with AngII (*D*) or irbesartan+AngII (*E*) for the indicated time. Immunoprecipitation (IP) and Western blot (WB) analyses of proteins extracted from mammary glands were performed for p-STAT3 and STAT3 (*A*, *B*, *D*) and p-ERK1/2 and ERK1/2 (*C*, *E*); 24-h involution mammary gland was included as positive STAT3 activation control. Results show 1 representative of 3 independent experiments performed. *F*) Immunohistochemistry using antibodies against STAT3 in lactating glands treated with AngII or vehicle. Lactating and 24-h involuted glands were included as negative and positive controls, respectively. Arrows indicate positive STAT3 translocation to the nucleus. Results show 1 representative experiment of 3. *G*) TUNEL analysis of mammary sections from lactating mice treated with AngII or vehicle for 8 h. Arrows indicate apoptotic cells (×400 view). *H*) Bars represent mean ± SE percentage of cells stained positive; $n = 3$ mice/group. $^{**}P < 0.01$ *vs.* control.

times the apoptotic nuclei were found in the glands of AngII-treated mice compared to the vehicle-treated mice (Fig. 2*G*, *H*). The finding that AngII induced STAT3 activation and apoptosis in lactating mammary epithelial cells *in vivo* prompted us to study its role in postlactational regression.

RAS components are expressed during mammary gland involution

We determined the expression pattern of AT_1, AT_2, AGT, and ACE throughout an entire lactating/involuting cycle in mouse mammary glands by qRT-PCR (**Fig. 3*A***). We found that AT_1 mRNA increased gradually, starting as soon as 24 h after removal of pups, maintaining this pattern for the following 3 d. AT_2 started to increase later than AT_1, and a significant increase was only detected at 96 h after removal of pups. AGT mRNA showed a significant increase at 48 h and even higher expression at 96 h. On the other hand, ACE exhibited a very fast increase in mRNA, with maximal expression 6 h after forced weaning. In addition, we confirmed by RPA that AT_{1A} expression was increased at 72–96 h of involution (Fig. 3*B*). Immunofluorescence analyses show protein expression of RAS components during mammary involution, which correlates with mRNA expression (Fig. 3*C*). As involution advanced, mammary glands exhibited higher staining for AT_1, AT_2, and AGT in epithelial cells and adipocytes, as well. The fact that there is a defined expression pattern of RAS components during postlactational regression suggests that these proteins are tightly regulated along this process and that they may play a significant role during mammary involution.

AT_1 receptor blockade resulted in reduced apoptosis and delayed involution

To define the role of endogenous AngII during mammary gland involution and to identify the receptor mediating the effects triggered by this peptide, *in vivo* experiments were performed using AT_1 receptor blockers. Lactating females and their litters were divided into 2 experimental groups. One group received subcutaneous injections in the back of Los, an AT_1 receptor blocker, while the second group received 0.9% NaCl (vehicle). At 24 and 48 h after initiation of involution, both groups showed similar histoarchitectural structures in the involuting mammary glands (**Fig. 4*A***). At 72 h after forced weaning, Los- and vehicle-treated glands had undergone extensive tissue remodeling, characterized by the collapse of the secretory lobuloalveolar structures. However, the Los group showed a mild persistence of open alveolar structures and slower reappearance of adipocytes in comparison to the vehicle group. By 96 h involution, differences deepened even more between the two groups. Los-treated group showed persistent collapsed lobuloalveolar structures with cumulous of epithelial cells, correlating with a decreased adipocyte population in these glands in comparison to vehicle-treated mice. These differences persisted at 120 h involution, clearly showing delayed involution in the glands of the Los group. Interestingly, we found significantly lower morphologically apoptotic

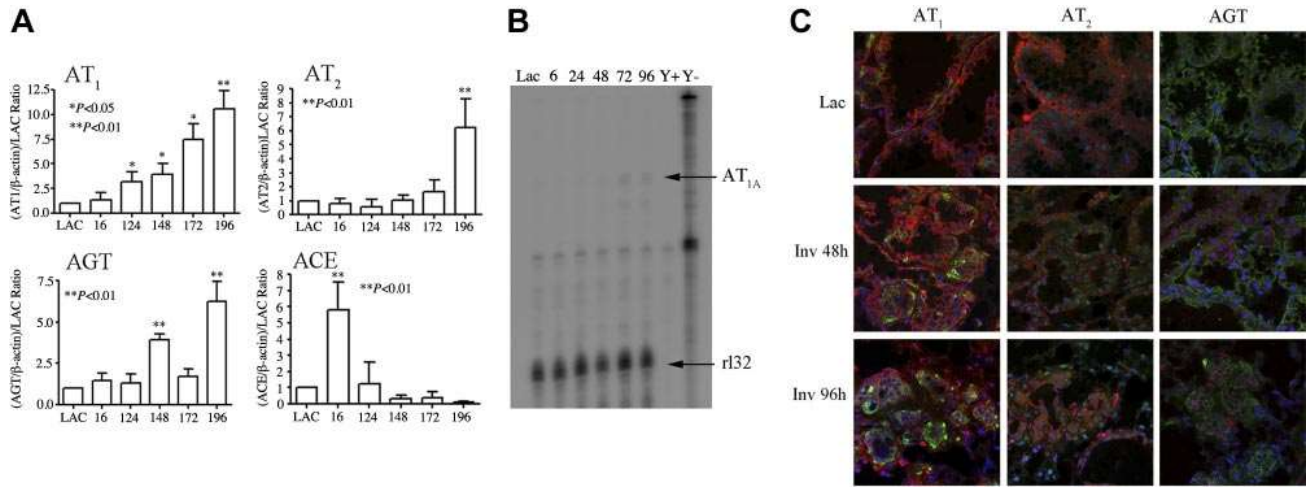

Figure 3. AT_1, AT_2, AGT, and ACE expression is modulated in lactating and involuting mouse mammary glands *in vivo*. *A*) qRT-PCR for the RAS components. RNA was extracted from mammary glands harvested at different time points during lactation and involution. qRT-PCR analysis was performed using the primers indicated. RNA levels are expressed as arbitrary units and have been normalized to actin and relative to lactation. Bars indicate means ± SE; $n \geq 4$. *B*) RNase protection assay for AT_{1A} receptor. The rl32 probe was included as a loading control. Results show 1 representative gel of 2 performed. In each experiment, mRNA of 3 mammary glands from independent experiments was pooled at each time point studied. *C*) Immunolocalization of RAS components in lactating and involuting mammary glands sections by 48 and 96 h. E-cadherin labeling was used to identify epithelial cells (AT_1 and AT_2, green; E-cadherin, red; AGT, red, E-cadherin, green; DAPI-stained nuclei, blue). Samples were frozen, cut with a cryostat, fixed, blocked, and incubated with the primary antibody indicated. Appropriate second antibody was added. Sections were analyzed under a laser confocal microscope. Results show 1 representative experiment of 3 (×100 view). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* lactation.

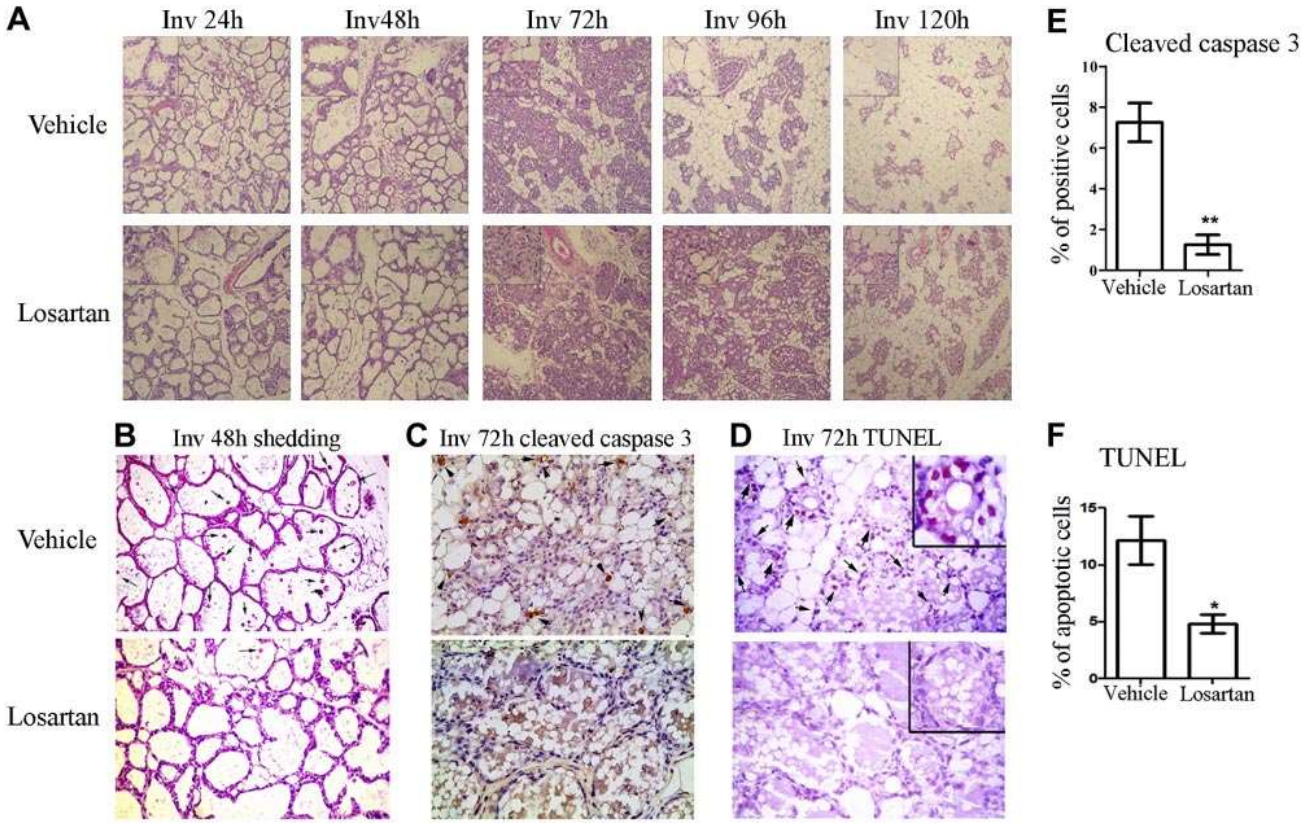

Figure 4. Morphological and molecular changes in mammary glands of Los-treated mice during involution. *A*) H&E-stained mammary gland sections at 24, 48, 72, 96, and 120 h involution from mice treated with vehicle (top) or Los (bottom) (×100 view; insets, ×400). *B*) Shedding of morphologically apoptotic epithelial cells into the alveoli lumen (indicated by arrows), at 48 h involution in vehicle- and Los-treated mice (top and bottom panels, respectively). *C*) Immunohistochemical assay against activated caspase-3 at 72 h involution in vehicle and Los-treated mice. *D*) TUNEL staining of mammary gland sections at 72-h involution from mice treated with vehicle (top) or Los (bottom) (×250 view; insets ×400). Bars represent mean ± SE percentage of cells stained positive for cleaved caspase 3 (*E*) and TUNEL (*F*); $n = 3$ mice/group. $^*P < 0.05$ and $^{**}P < 0.001$ *vs.* control.

epithelial cells accumulated in the open lumen of the alveoli at 48 h involution in the Los-treated mice (Fig. 4*B*). The antiapoptotic effect of Los on epithelial cells was then assessed by immunohistochemical assays against activated cysteine-dependent aspartate-specific proteases 3 (caspase-3; Fig. 4*C*, *E*) and TUNEL (Fig. 4*D*, *F*). As expected, the percentage of apoptotic cells found in mammary glands of Los-treated mice was significantly lower when compared to vehicle-treated mice at 72 h involution.

Taken together, these results show that AT_1 blockade during involution resulted in epithelial apoptosis inhibition and involution delay.

AT_1 receptor antagonism affects involution remodeling process

In an attempt to identify molecular mechanisms underlying the delay in involution observed after disruption of AT_1-mediated signaling, we studied critical signaling pathways associated with apoptosis during involution. As shown in **Fig. 5*A***, we did not observe significant alterations in the phosphorylation levels of the proapoptotic factors STAT3 and ERK1/2 in mammary glands at any of the examined time points of involution after Los treatment. Interestingly, BCL-x_L, an antiapoptotic member of the Bcl-2 family, was found to be more strongly expressed in mammary glands at 24 and 48 h after weaning in Los- compared to vehicle-treated mice. AKT has been proposed as a potent survival signal for the involuting mammary gland (18). We found a substantial increase of phosphorylated levels of AKT at 24 and 48 h after forced weaning in Los-treated mice. With the aim to elucidate whether AT_1 receptor blockade alters the expression of early responsive genes during involution, we analyzed the expression of LIF and TNF-α. Thus, mammary glands from mice treated with Los or vehicle during involution were studied for the expression of LIF and TNF-α mRNA by qRT-PCR. A significant inhibition of both locally produced factors was observed in the Los-treated mice (Fig. 5*B*). Thus, AT_1 blockade during involution induces pAKT and BCL-x_L increase, resulting in epithelial apoptosis inhibition and involution delay.

The second phase of mammary involution is irreversible and involves breakdown of the ECM and tissue remodeling that is mostly generated by the

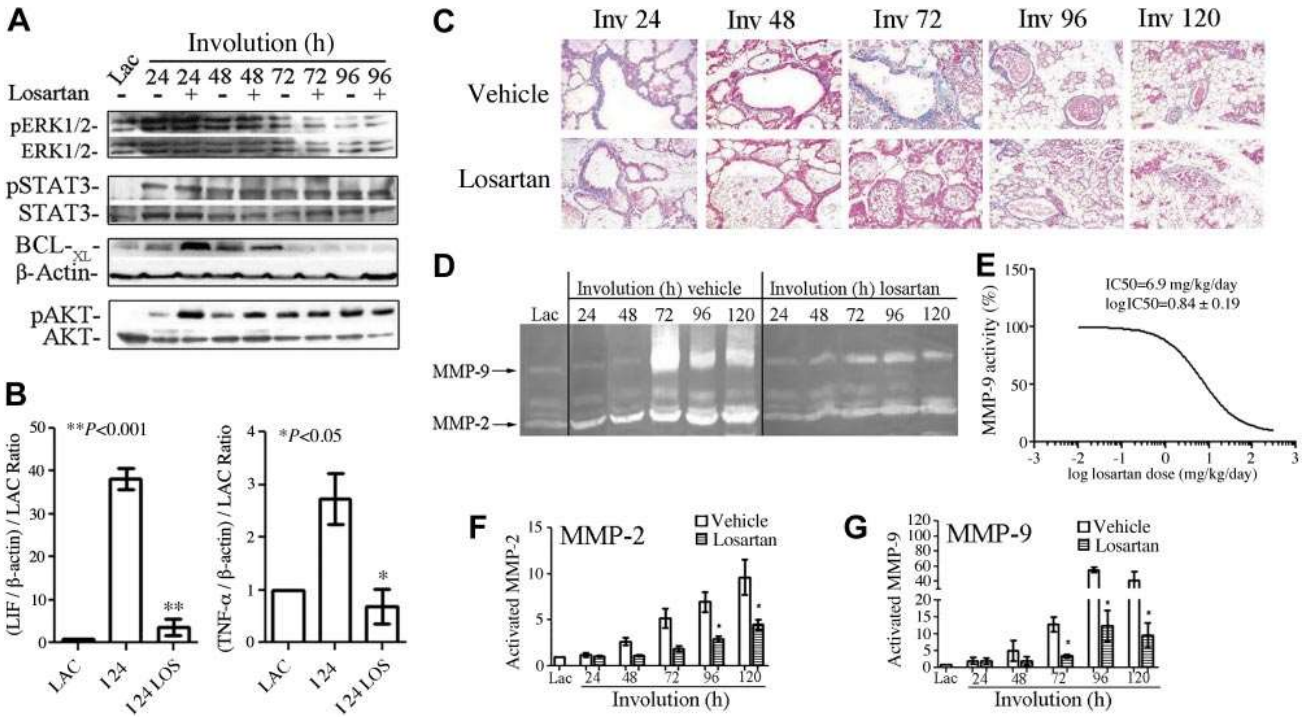

Figure 5. Los inhibits collagen deposition and MMP-2 and MMP-9 activities during involution. *A*) Western blot analyses of proteins extracted from mammary glands of Los- *vs.* vehicle-treated mice during involution were performed for phosphorylated and native forms of ERK1/2 and STAT-3 and for AKT, BCL-x_L, and β-actin. Results show 1 representative experiment of ≥3 performed. *B*) qRT-PCR analysis was performed for LIF and TNF-α. RNA was extracted from mammary glands 24 h after weaning from mice treated with Los or vehicle during involution, RNA levels are expressed as arbitrary units and have been normalized to β-actin and relative to lactation. Bars indicate means ± SE; $n = 3$. *C*) Masson's Trichrome staining of mammary glands sections, from mice treated with vehicle (top) or Los (bottom) at different time points of involution (blue, collagen and reticulin fibers; dark red/purple, nuclei; red/pink, cytoplasm). Results show 1 representative experiment ($n \geq 4$; ×250 view). *D*) Zymographic analysis of MMP-2 and MMP-9 activities in mammary glands from vehicle- *vs.* Los-treated mice at 24, 48, 72, 96, and 120 h involution. Results show a representative negative image of a gelatin zymogram for lactation and involuted glands of ≥3 independent experiments performed. *E*) Log (inhibitor) *vs.* response curve kinetics for MMP-9 activity at 96 h involution were carried out using different daily doses of Los to determine the $\log IC_{50}$ and IC_{50} values shown in the graphic. Los inhibited MMP-9 activity in a dose-dependent manner. Experiment was performed in triplicate; values represent means ± SE. *F*, *G*) Densitometric analyses of zymograms for MMP-2 (*F*) and MMP-9 (*G*), as described in *D*. Values are expressed as means ± SE; $n \geq 3$. $^*P < 0.05$ $^{**}P < 0.001$ *vs.* control.

activity of metalloproteases (36). Taking advantage of Masson's Trichrome stain, which allows visualization of collagen and reticular fibers of connective tissue, we analyzed these components of the mammary stroma during involution in Los-treated mice. As shown in Fig. 5*C*, less collagen and reticulin deposition fibers were found around alveolar and ductal structures of mammary sections from Los-treated compared to vehicle-treated mice at 48, 72, and 96 h after weaning. Zymographic analyses of MMP-2 and MMP-9 in glands from Los- *vs.* vehicle-treated mice identified inhibition in both metalloprotease activities in the late phase of involution (72, 96, and 120 h; Fig. 5*D*). Los treatment during involution inhibited MMP-9 activity in a dose-dependent manner. Los IC_{50} values and corresponding maximal and minimal effects from dose-response curves at 96 h involution were calculated (Fig. 5*E*). As shown in Fig. 5*F, G*, AT_1 receptor blockade during involution significantly inhibited MMP-2 and MMP-9 activities, respectively. These data suggest that endogenously generated AngII acting through AT_1 may induce MMP activation and collagen deposition, hallmarks of the late phase of mammary gland involution.

Mice deficient in AT_{1A} and/or AT_{1B} receptor isoforms show delayed involution

Rodents carry two isoforms of the AT_1 receptor, AT_{1A} and AT_{1B}, which are products of differentially expressed and regulated genes and are pharmacologically indistinguishable (8). To investigate the relevance of AT_1 receptor isoforms during involution, we analyzed mammary glands from AT_{1A}-, AT_{1B}-, and AT_{1A}/AT_{1B}-deficient mice. We found that lactation was not substantially perturbed as pups, born to AT_1-deficient mothers and WT fathers to prevent possible effects of AT1-deficient pups, fed and grew normally. Accordingly, histoarchitectural morphology of lactating glands from AT_1 single- and double-deficient females showed no evident variations compared to WT animals. However, the absence of AT_1 had dramatic effects on involution.

Consistent with the results obtained with the AT_1 antagonist Los during involution, both AT_{1A}- and AT_{1B}-deficient mice showed a significant delay in involution, implicating that both isoforms play a role during this process. By 72 h after weaning, WT gland shows scattered cumulous of epithelial cells with intense adipose tissue invasion (**Fig. 6*A***). In contrast, AT_{1A}-deficient mice showed almost all lobuloalveolar structures open and distended with little evidence of collapse and minimal adipose tissue invasion. At the same time point of involution, AT_{1B}-deficient mice showed similar but milder changes than AT_{1A}KO glands. By 96 h involution, AT_1-deficient glands showed persistent and open alveolar structures with a higher epithelial/adipocyte cell ratio compared to the WT at the same time point. As shown in Fig. 6*B*, mammary glands from AT_{1A}/AT_{1B} DKO mice at 72 h involution, showed reduced collagen deposition around ductal and alveolar structures when compared to WT. Notably, the involution delay observed in AT_1-knockout mice correlated with a significantly lower rate of apoptotic epithelial cells at 72 h of postlactational regression assessed by TUNEL (Fig. 6*C, D*) and immunohistochemical assays against activated caspase-3 (Fig. 6*E, F*). Mammary glands from AT_{1A}- and/or AT_{1B}-deficient mice showed an even more striking delay in involution when compared to glands from Los-treated WT mice. This strongly supports the idea that AngII, acting through both AT_1 receptor isoforms, plays a critical role in apoptosis and remodeling during this phase.

DISCUSSION

Using AT_1 receptor blockers and models of genetic AT_1 deficiency, we identified the interruption of AT_1 signaling as inducer of survival factors like BCL-x_L and AKT, and as inhibitor of MMP-2 and MMP-9, all key players in involution. AT_1-deficient mice showed reduced rate of epithelial apoptosis, retarded stromal invasion, and remodeling with massive delay in mammary gland involution. These results strongly support the existence of a functional tissue-specific mammary RAS. We hypothesize that locally generated AngII acting *via* its AT_1 receptor, plays a critical role in mammary gland involution.

During the past decades, it has been demonstrated that RAS components are expressed in almost every tissue studied (4), and new organ-specific functions of local RAS have been described (37). AT_1 was found to be especially abundant in secretory epithelial tissues (38). Recently, the existence of a local RAS in the mammary gland has been postulated (19, 20, 22), and it has been hypothesized that it could directly or indirectly contribute to breast cancer progression (21, 39). Moreover, several studies demonstrate the potential antitumor effects of AT_1-receptor blockers in cancer (40–42). Nevertheless, our present work is the first demonstration and a significant proof that a local RAS has actually a functional role in normal mammary gland physiology.

Postlactational regression is a complex multistep process with a wound-healing signature and mild inflammation, both associated with breast cancer progres-

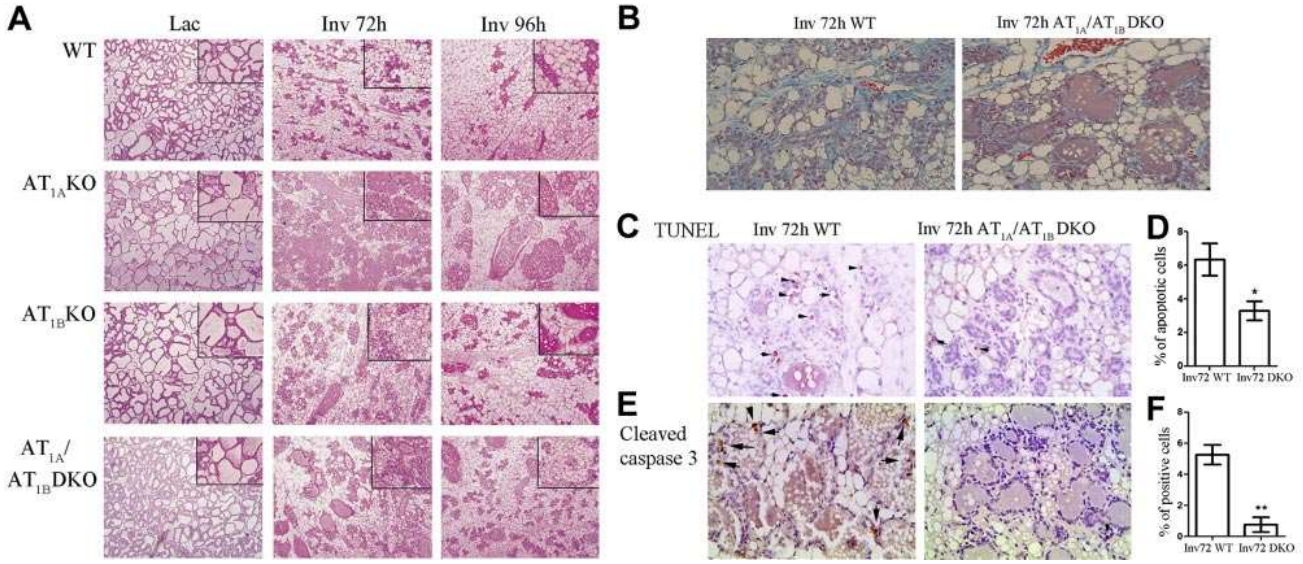

Figure 6. AT_1-deficient mice show delayed involution. *A*) H&E-stained sections of lactating and involuting (72 and 96 h) mammary glands from WT, AT_{1A}KO, AT_{1B}KO, and AT_{1A}/AT_{1B} DKO mice (×100 view; insets, ×400). *B*) Masson's Trichrome-stained sections of involuting mammary glands at 72 h after removal of pups from WT and AT_{1A}/AT_{1B} DKO mice (blue, collagen and reticulin fibers; dark red/purple, nuclei; red/pink, cytoplasm). Results show 1 representative experiment of 3 (×400 view). *C*) TUNEL staining of mammary gland sections at 72-h involution from WT and AT_{1A}/AT_{1B} DKO. Arrows show representative apoptotic cells (×400 view). *D*) Bars represent mean ± SE percentage of positive stained cells; $n = 3$ mice/group. *E*) Immunohistochemical assay against activated caspase-3 at 72-h involution in WT and AT_{1A}/AT_{1B} DKO mice. Arrows indicate representative positive cells. *F*) Bars represent mean ± SE percentage of positive stained cells for cleaved caspase-3; $n = 3$ mice/group. $^*P < 0.05$ $^{**}P < 0.001$ *vs.* WT.

sion, metastasis, and survival (43). In fact, there is an increased interest in mammary gland involution, not only for being a useful model for studying developmentally regulated apoptosis and tissue remodeling, but also because, paradoxically, many factors that induce apoptosis and remodeling during involution, such as NF-κB, metalloproteases, or STAT3, are also constitutively active in breast cancer (44). Therefore, we postulate a functional association of local RAS not only with mammary involution, but also with breast cancer development.

In this study, we show that not only AT_{1A} but also AT_{1B} disruption induces delay of mammary gland involution. By using mice with targeted disruption of the AngII receptor subtype 1A gene (*Agtr1a*) or the AngII receptor subtype 1B gene (*Agtr1b*), we aimed to distinguish the relative contribution of each AT_1 isoform during mammary gland regression. The isoforms share substantial DNA sequence homology but differ in distribution and regulation among tissues and cell types (9). Because they are pharmacologically indistinguishable, gene targeting was an alternative approach to clarify the functional significance of these two distinct angiotensin AT_1 receptor subtypes *in vivo*. In rodents, AT_{1A} mediates most of the known actions of AngII in the cardiovascular and renal systems (8). However, little is known about the function of the AT_{1B} receptor in cells that express this receptor subtype. It has been suggested that in rodents, the AT_{1B} may be redundant and share functional properties with the AT_{1A} receptor (45). On the other hand, growing evidence supports the notion that AT_1 receptor subtypes differ not only in distribution and regulation, but also in their function (46). Here, we describe the novel findings that AT_{1A} and AT_{1B} are expressed in the gland and that both isoforms play a redundant function in postlactational regression. Nevertheless, AT_{1A} seems to play a more critical role, judging for the more drastic histoarchitectural phenotype in the AT_{1A}KO gland during involution.

It has been demonstrated that STAT3 plays a crucial role in initiating apoptosis at the onset of mammary regression. Local factors stimulate STAT3 phosphorylation and translocation to the nucleus during involution (12). Mammary glands of STAT3 conditional-knockout mice showed suppression of epithelial apoptosis that led to a dramatic delay in mammary gland involution. We and others previously reported that LIF and IL-6 activate STAT3 during involution (13, 33, 47). The fact that AngII induced STAT3 activation in the nontransformed murine mammary epithelial cell line HC11 prompted us to determine whether AngII could trigger STAT3 phosphorylation *in vivo*. Lactation is the ideal phase to study factors responsible for triggering STAT3 activation, since it is normally inactive during this phase. We found an increased level of STAT3 phosphorylation and nuclear translocation in the AngII-treated lactating mammary gland identifying AngII as a new putative local factor involved in STAT3 activation in mammary glands. Moreover, this activation was mediated by AT_1, as it was blocked by the cotreatment with irbesartan, an AT_1 receptor blocker. Since ACE expression peaks 6 h after removal of pups, we believe that AngII could be locally generated in the early phase of involution, triggering STAT3 activation in collaboration with other local factors, usch as LIF and IL-6, thereby inducing apoptosis. These results led us to study the role of AT_1 receptor during involution when STAT3 is known to be activated. We found that while involution delay induced by AT_1 receptor blockade was independent from the status of activation of the proapoptotic factor STAT3, it could be explained by the activation and/or induction of survival factors like AKT and BCL-x_L and the inhibition of the early response factors LIF and TNF-α. Nevertheless, we cannot exclude the possibility that AngII acting through AT_1, together with other local factors, mediates STAT3 activation during involution. The fact that pSTAT3 levels remained intact despite AT_1 receptor blockade during involution could indicate that many local factors, such as LIF or IL-6, and possibly AngII, as postulated in this work, have redundant functions regarding STAT3 activation, thus compensating the putative inhibitory effect of AT_1 receptor blockade on STAT3 activation.

The increase of antiapoptotic and survival signals in the postlactating mammary glands treated with the AT_1 receptor blocker is a key molecular mechanism underlying the delay in mammary gland involution observed in this experimental setting. The PI3K/AKT pathway and members of the Bcl-2 family have been identified as key regulators of apoptosis/cell survival in mammary glands (17, 18, 43). It has been reported that during involution, constitutively active *Akt* transgene provides an overriding survival signal (18), while deletion of the antiapoptotic BCL-x_L gene accelerates apoptosis (16, 17). Our results suggest that AngII acting through AT_1 receptor induces apoptosis in involuting mammary glands, possibly through the down-regulation of pAKT and BCL-$_{XL}$.

The other critical underlying mechanism unveiled here is the inhibition by the AT_1 receptor blocker, Los, of the expression of proapoptotic mediators, such as LIF and TNF-α, during involution. Initially, removal of pups and milk stasis cause induction of local factors that lead to epithelium apoptosis. It has been shown that LIF and TNF-α expression are induced only a few hours after lactation interruption (14, 33). The significantly lower expression of these local factors at early stages of involution, induced by AT_1 receptor blockade, could, in part, explain the involution delay.

We found specific expression kinetics of AT_1, AT_2, ACE, and AGT during lactation-involution phases in mouse mammary glands. During lactation, RAS expression was relatively low. Interestingly, the expression of AT_1, AT_2, and AGT rose during the late phase of mammary involution, while ACE peaked as early as 6 h after removal of pups, strongly suggesting a role for the RAS during mammary regression. It has been proposed that the primary stimulus for triggering epithelial apoptosis

originates from local mammary factors (12). A number of potential mediators of this stimulus have been proposed, including mechanical stretch of the epithelium secondary to milk stasis and alveolar distention (29). Cytokines from the IL-6 family are known to increase their expression after mechanical stretch in different cell types (48, 49). It has been widely demonstrated that mechanical stretch can trigger the expression of all gene transcripts of the RAS *in vitro* and *in vivo* too (23, 50). Interestingly, AT_1 receptor blockade abolished the intracellular pathways activated by the induction of the local RAS secondary to mechanical stretch in different tissues (51, 52). Mechanical stretch signal transduction in rat myocardiocytes included activation of the JAK/STAT pathway (48). It has also been suggested that AT_1 receptors could be activated by mechanical stretch in the absence of their ligand, AngII (53). We hypothesize that mechanical stretch induced by the lack of suckling, could be triggering ACE expression during the early phase of mammary regression, increasing the local production of AngII. The octapeptide, acting in a paracrine/autocrine fashion together with other local factors, could then activate the JAK/STAT3 pathway, inducing apoptosis. Nevertheless, more experiments should be done to discriminate whether mechanical stretch directly triggers AT_1 activation and/or induces RAS expression in mammary epithelial cells. It also remains to be elucidated what are the stimuli that trigger AT_1, AT_2, and AGT expression in the late phase of involution.

The delay in involution induced by AT_1 receptor blockade was characterized by retarded alveolar collapse, collagen deposition, and adipocyte invasion. The significant inhibition on MMP-2 and MMP-9 activities found after blocking AT_1 receptor could explain the delay in the ECM remodeling pattern and show the relevance of AT_1 signaling in MMP activation during the late phase of mammary involution. During the second phase of postlactational regression, the activity of MMP-2, MMP-3, and MMP-9 is increased (15, 44). MMP activity contributes to epithelial cell loss and remodeling of the mammary tree by disrupting critical epithelial-ECM interactions. Alveolar structures start to collapse, and adipocytes begin to repopulate the gland (36). However, little is known about the signaling pathways that mediate MMP activation, collagen deposition, and adipose tissue regeneration during the mammary involution process. AngII is known to participate not only in inflammation but also in tissue remodeling processes. It has been demonstrated that AngII induces the expression and activity of metalloproteases in different cell types (25, 54, 55) It has also been reported that AT_1 receptor blockers diminish collagen deposition during tissue remodeling in different models of disease, supporting the findings reported herein (42, 56).

Considering that the histological structure of the mammary gland shows two main populations of cells, epithelial cells and adipocytes, we cannot exclude the possibility that disruption of AT_1 receptor signaling may be having consequences on both cell types, especially during the second phase of involution when adipocyte population grows. Here, we show that mammary gland adipose tissue expresses RAS components. Interestingly, involuted mammary glands from AT_1 receptor-knockout mice or Los-treated mice show a significantly slower tissue replacement with adipocytes compared to controls. Regarding adipose tissue, it expresses all components of the RAS necessary to generate angiotensin peptides for local function. Preadipocytes and adipocytes express both receptors, AT_1 and AT_2 (57). It has also been demonstrated that AngII induces expression of cyclin D1 mRNA and G_1 phase progression in human preadipocytes and regulates adipocyte differentiation (58). We postulate that in the mammary gland, local RAS could have two different sources: the epithelial component and the adipose tissue component and that it could affect both cell types regulating proliferation, differentiation, and survival.

The role of AngII on mammary gland physiology has not been explored before. Our present work is the first demonstration that a local RAS actually has a functional role in the mammary gland. By two different approaches, pharmacological blockade of AT_1 and targeted disruption of *Agtr1a* or *Agtr1b*, we demonstrate that AngII, acting through both AT_1 receptor isoforms, plays a critical role in postlactational regression. FJ

The authors thank Dr. Monica Vermeulen for helping with *in vivo* experiments, Dr. Nancy Hynes (Friedrich Meischer Institute, Basel, Switzerland) for providing the HC11 cell line, and Dr. Omar Coso (University of Buenos Aires) for kindly providing some of the antibodies used for signal transduction studies. The authors are grateful to Beatriz Loria for excellent technical assistance. This work was supported by research grants awarded to K.N. from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT; PICT 2008-1979) and Roemmers Foundation (2009); to C.S.L. from ANPCyT (PICT 31948) and Consejo Nacional de Investigaciones Cientificas y Tecnicas (PIP 112-200801-00859); to T.W. and C.S.L. from Ministerio de Ciencia, Tecnología e Innovación Productiva–Deutscher Akademischer Austausch Dienst (DA0809); and to M.S. from ANPCyT (PICT 00325) and the Susan G. Komen for the Cure Foundation (BCTR0600341). The authors declare no conflicts of interest.

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Received for publication August 5, 2011.
Accepted for publication January 10, 2012.