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Angiotensin II suppresses adriamycin-induced apoptosis through activation of phosphatidylinositol 3-kinase/Akt signaling in human breast cancer cells

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Angiotensin II (Ang II) stimulates tumor growth and angiogenesis in some solid cancer cells, but its anti-apoptosis role in breast cancer remains unclear. To address this issue, we investigated the effect of Ang II on adriamycin-induced apoptosis in breast cancer MCF-7 cells. Treatment of human breast cancer MCF-7 cells with adriamycin, a DNA topoisomerase IIα inhibitor, caused apoptosis. However, cells pretreated with Ang II were resistant to this apoptosis. Ang II significantly reduced the ratio of apoptotic cells and stimulation of phospho-Akt-Thr308 and phospho-Akt-Ser473 in a dose-dependent and time-dependent manner. In addition, Ang II significantly prevented apoptosis through inhibiting the cleavage of procaspase-9, a major downstream effector of Akt. The Ang II type 1 receptor (AT1R) was responsible for these effects. Among the signaling molecules downstream of AT1R, we revealed that the phosphatidylinositol 3-kinase/Akt pathway plays a predominant role in the anti-apoptotic effect of Ang II. Our data indicated that Ang II plays a critical anti-apoptotic role in breast cancer cells by a mechanism involving AT1R/phosphatidylinositol 3-kinase/Akt activation and the subsequent suppression of caspase-9 activation.

Keywords angiotensin II; angiotensin II type 1 receptor; PI3-kinase/Akt; anti-apoptosis; breast cancer

Angiotensin II (Ang II), a multifunctional bioactive octapeptide of the rennin-angiotensin system, plays a fundamental role as a vasoconstrictor in controlling cardiovascular function and renal homeostasis. Ang II acts as a potent growth factor and cytokine in vascular smooth muscle cells [1], cardiac myocytes [2], and cardiac fibroblasts [3]. Recently, it has been shown that Ang II stimulates tumor growth and angiogenesis, including choriocarcinoma [4], breast cancer [5], ovarian cancer [6], and pancreatic cancer [7], suggesting that Ang II might also be involved in cancer development.

Ang II mediates its biological effects through binding to two subtypes of receptors, the Ang II type 1 receptor (AT1R) and the Ang II type 2 receptor (AT2R), which belong to the superfamily of G-protein-coupled receptors [8]. The major functions of Ang II in tumor growth and angiogenesis are mediated through AT1R [9–12].

Inhibition of apoptosis and cellular proliferation are important mechanisms in carcinogenesis. Ang II also activates multiple signaling pathways related to cell apoptosis and proliferation, including protein kinase C [4,13] and mitogen-activated protein kinase [14]. Recently, stimulation of AT1R has been shown to trigger the activation of phosphatidylinositol 3 (PI3)-kinase and Akt [15]. It has been reported that the PI3-kinase pathway is an important anti-apoptotic signal pathway that is frequently activated in cancer cells [16,17]. The most important downstream effector of PI3-kinase is the serine/threonine kinase Akt or protein kinase B. The induction of Akt activity is primarily under the control of phosphoinositide products of PI3-kinase, PIP2, and PIP3, which bind to the pleckstrin homology domain of membrane-associated Akt. Subsequently, full activation of Akt requires phosphorylation of two amino acids in Akt, one within the activation loop (Thr308) and one at the C-terminus hydrophobic domain (Ser473) [18,19]. Activated Akt phosphorylates pro-apoptotic proteins, including Bad, caspase-9, and forkhead transcription factors [20–22], thereby inhibiting apoptosis. Ang II has been reported to significantly prevent cisplatin-induced apoptosis through nuclear factor-κB activation and the subsequent production of anti-apoptotic molecules, including survivin and Bcl-XL, in pancreatic cancer cells [7]. However, with regard to breast cancer cells, little is known about the effect of Ang II in

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apoptosis. In this article, we studied the potential roles of Ang II on adriamycin (ADR)-induced apoptosis, and found that Ang II plays an anti-apoptotic role in MCF-7 cells. We further revealed the underlying molecular mechanisms by examining the effects of Ang II on signaling pathways related to apoptosis. The signaling molecules include AT1R, PI3-kinase, and Akt. We further showed that caspase-9 participates in this anti-apoptotic effect of Ang II downstream of Akt.

Materials and Methods

Reagents and antibodies

Human Ang II was purchased from AnaSpec (San Jose, USA). AT1R antagonist, losartan (DuP 753), was purchased from DuPont Merck (Hangzhou, China). AT2R antagonist, PD123319, was purchased from Sigma-Aldrich (St. Louis, USA). LY294002, Akt, phospho-Akt-S473, phospho-Akt-T308, and procapase-9 antibodies were from Cell Signaling Technology (Boston, USA).

Cell lines and culture

MCF-7 cell lines were the generous gift of Heilongjiang Cancer Institute (Harbin, China). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 U/ml penicillin/50 μg/ml streptomycin, then incubated at 37 ºC in a humidified atmosphere of 5% CO_2.

Apoptosis morphological analysis

Serum-starved MCF-7 cells were incubated with or without Ang II and/or losartan and the chemotherapeutic agent ADR for 24 h. Cells were collected, washed with phosphate-buffered saline (PBS), and stained with an equal volume of Hoechst 33342 and propidium iodide (PI) for 20 min at 4 ºC. Then the cells were washed gently with PBS. The morphology of the nuclei was examined using a fluorescence microscope (Olympus, Tokyo, Japan).

DNA fragmentation analysis

Fragmented DNA was extracted with a DNA purification kit (Beyotime Biotechnology, Hangzhou, China). A total of 10^7 cells were collected and suspended in 200 μl PBS, 4 μl Rnase A, and 20 μl Proteinase K, and incubated for 2 min at room temperature. Then 200 μl sample lysis buffer B was added and incubated for 10 min at 70 ºC. The sample was mixed with 200 μl dehydrated alcohol and pipetted into a filter tube. The mixture was centrifuged at 6000 *g* for 1 min and the flow-through was discarded. Washing buffer I (500 μl) was added to the filter tube, then centrifuged as above. Washing buffer II (600 μl) was added to the filter tube and centrifuged at 18,000 *g* for 1 min, then the flow-through was discarded. The washing step was repeated, then a final high speed spin (18,000 *g*) was carried out for 1 min. One hundred microliters of warm elution buffer was added to the filter tube. The eluted DNA was collected by centrifugation at 18,000 *g* for 1 min, and analyzed electrophoretically on 1% agarose gels containing 0.1% ethidium bromide. The DNA band patterns were visualized under ultraviolet illumination.

Flow cytometry

MCF-7 cells were incubated with or without Ang II and/or losartan and the chemotherapeutic agent ADR. The adherent cells were collected by trypsin, combined with floating cells, and centrifuged at 1000 *g* for 5 min. After being washed with PBS, cells were added into 500 μl of annexin V binding buffer (Keygen, Nanjing, China), and incubated for 10 min with 5 μl fluorescein-isothiocyanate-conjugated annexin V and 5 μl PI (Keygen), followed by two-color flow cytometric analysis (Beckman Coulter, Fullerton, USA). Fluorescence was measured with a minimum of 10,000 events for each sample in a fluorescence-activated cell sorter according to the method suggested by the manufacturers. For each cell, fluorescence channels 1 (annexin V-fluorescein-isothiocyanate) and 3 (PI) were recorded. Annexin V-positive cancer cells that did not take up PI were identified as early apoptotic cells, whereas doubly positive cancer cells were classified as late apoptotic cells.

Western blot analysis

MCF-7 cells were lysed in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. After centrifugation at 12,000 *g* for 5 min at 4 ºC, the supernatant was obtained. The supernatant was used as a total cell lysate and analyzed for protein concentration by the Bradford method (BioRad, Hercules, USA). Equal amounts of cellular proteins (30 μg/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 1% skimmed milk in Tris-buffered saline/Tween 20 overnight at 4 ºC, the blots were incubated with rabbit anti-human Akt, phospho-Akt-S473, phospho-Akt-T308, or procapase-9 antibody at a dilution of 1:600 for 2 h at room temperature. The blots were subsequently washed three times (10 min for each wash) with Tris-buffered saline/Tween 20 then incubated with the appropriate alkaline phosphatase-conjugated anti-rabbit secondary antibody (dilution 1:5000; Promega, Madison, USA) for 1 h at room temperature. The bands were visualized using the BCIP/NBT (Promega) coloration method.

Statistical analysis

Data are expressed as the mean±SD. Statistical analysis was carried out using Student's *t*-test for unpaired samples and ANOVA. A *P* value less than 0.05 was considered statistically significant.

Results

Ang II blocks apoptosis in MCF-7 cells through AT1R

To study the effect of Ang II on anti-apoptosis, MCF-7 cells were exposed to 0.1 μM ADR, a chemotherapeutic agent commonly used to treat patients with breast cancer. It was anticipated that approximately 30% of the cells would be killed. We detected apoptosis through observation of nuclear morphology by staining cells with Hoechst 33342 and PI. The cells showed a distinct condensed and fragmented chromatin in the nuclei after treatment with 0.1 μM ADR for 24 h, but the characteristics for apoptosis were markedly suppressed by Ang II pretreatment in a dose-dependent manner [**Fig. 1(A)**]. One hundred nanomoles of Ang II had the maximal inhibition effect (P <0.01). Cells were further subjected to nuclear DNA fragmentation analysis [**Fig. 1(B,C)**]. Ang II pretreatment clearly inhibited ADR-induced DNA fragmentation. These results were further confirmed by PI/annexin V [**Fig. 1 (D,E)**]. The percentage of cancer cells in the apoptotic phase was (30.17±2.17)% with 0.1 μM ADR. However, the percentage of apoptotic cells was significantly reduced by pretreatment with 100 nM Ang II (P<0.01). To determine the receptor responsible for mediating the anti-apoptotic effects of Ang II, we used receptor-type specific inhibitors. Losartan, an AT1R antagonist, completely suppressed the anti-apoptotic effect of Ang II, whereas PD123319, an AT2R antagonist, had no effect (**Fig. 1**). These results indicated that Ang II exerts its anti-apoptotic effect in MCF-7 cells through the AT1R. Therefore, cells were not pretreated with PD123319 in subsequent experiments.

Anti-apoptotic effect of Ang II in MCF-7 cells is mediated by PI3-kinase/Akt pathway

To further study the mechanisms underlying the anti-apoptotic effect of Ang II in MCF-7 cells, we investigated the signaling molecules in pathways downstream of AT1R. PI3-kinase/Akt is a key regulatory pathway that controls the cellular response to apoptosis. Therefore, we hypothesized that the anti-apoptotic effect of Ang II in MCF-7 cells might be one of the biological consequences of PI3-kinase/Akt activation. To address this possibility, MCF-7 cells were pretreated for 30 min with LY294002, a PI3-kinase inhibitor, and then stimulated with Ang II for 24 h. At 50 μM LY294002, the anti-apoptotic effect of Ang II was completely reversed (**Fig. 1**). These results indicated that PI3-kinase plays a predominant role in the anti-apoptotic effect of Ang II and prompted us to further examine the potential link between Ang II and Akt, a major downstream effector of PI3-kinase.

Western blot analyses revealed that Ang II stimulates phospho-Akt-Thr308 and phospho-Akt-Ser473 in MCF-7 cells in a dose-dependent and time-dependent manner. As shown in **Fig. 2(A)**, 100 nM Ang II caused maximal phosphorylation of Akt and induced a significant increase of Akt phosphorylation at 15 min. The peak level of phospho-Akt persisted for at least 6 h [**Fig. 2(B)**]. Therefore, these results suggested that Ang II exerts its anti-apoptotic effect in MCF-7 cells through PI3-kinase and the subsequent Akt activation.

Ang II stimulates phosphorylation of Akt through AT1/PI3-kinase

We also investigated the molecular mechanisms leading to Akt activation in Ang II signaling. It was revealed that the AT1R inhibitor losartan completely blocked the Ang II-induced phosphorylation of Akt. This indicated that AT1R is responsible for Ang II-induced Akt activation. Because PI3-kinase was reported to mediate Akt activation, we then tested the effects of LY294002. PI3-kinase inhibitors completely abolished Akt phosphorylation in response to Ang II (**Fig. 3**).

Ang II inhibits caspase-9 activation through AT1/PI3-kinase

Cardone *et al* [20] reported that Akt induced the phosphorylation of procaspase-9, thereby inhibiting its protease activity. Therefore, we further studied the effect of Ang II on caspase-9 activation after exposure of MCF-7 cells to ADR. As shown in **Fig. 4(A)**, ADR treatment decreased the procaspase-9 level and concomitantly caused the appearance of a cleaved caspase-9 fragment. However, Ang II pretreatment suppressed the ADR-induced cleavage of procaspase-9. The suppressive effects of Ang II on the cleavage of procaspase-9 were completely inhibited by pretreatment with losartan and LY294002, suggesting that this suppression is through a mechanism dependent on AT1R and PI3-kinase [**Fig. 4(B)**].

Discussion

Recent studies have shown that normal and cancerous

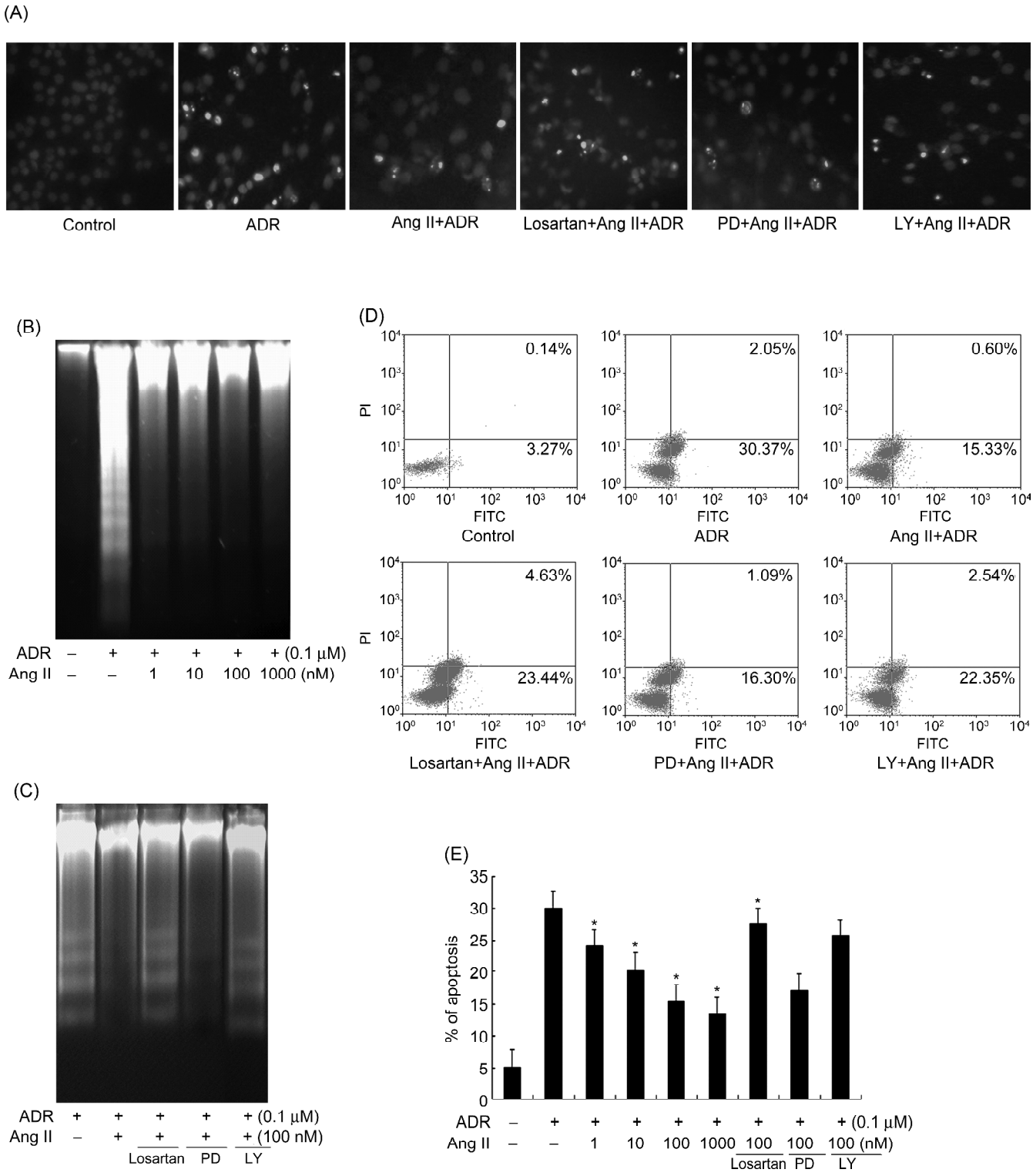

Fig. 1 Angiotensin II (Ang II) blocks apoptosis in human breast cancer MCF-7 cells through pathways mediated by Ang II type 1 receptor and phosphatidylinositol 3-kinase Cells were pretreated with either 10^{-5} M losartan or 50 μM LY294002 (LY) and incubated for 24 h in 0.1 μM adriamycin (ADR) serum-free medium with or without Ang II (1–1000 nM). (A) Hoechst 33342 and propidium iodide staining assay. The maximal inhibitory effect on ADR-induced apoptosis was achieved with 100 nM Ang II. Losartan or LY294002 completely suppressed the anti-apoptotic effect of Ang II, whereas PD123319 (PD), an Ang II type 2 receptor antagonist, had no effect. Magnification, 200×. (B,C) DNA laddering assay. (D,E) Flow cytometric analysis. Results shown are the mean±SD of three different experiments. *P<0.01 versus 0.1 μM ADR group.

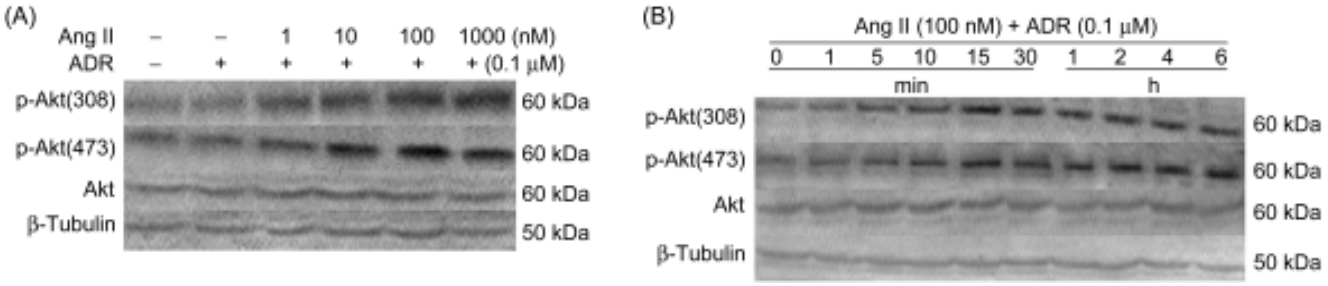

Fig. 2 Angiotensin II (Ang II) stimulates the activation of the Akt pathway in a dose-dependent and time-dependent manner (A) Dose-response study showing that 100 nM Ang II caused maximal phosphorylation of Akt. (B) Time course study showing that 100 nM Ang II induced a significant increase of Akt phosphorylation at 15 min. ADR, adriamycin; p-Akt(308), phospho-Akt-Thr308; p-Akt(473), phospho-Akt-Ser473. Beta-tubulin served as an internal control for normalization purposes.

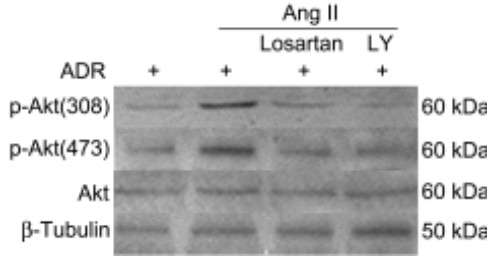

Fig. 3 Angiotensin II (Ang II) stimulates phosphorylation of Akt through pathways mediated by Ang II type 1 receptor and phosphatidylinositol 3-kinase Cells were pretreated for 30 min with control buffer, 10^{-5} M losartan, or 50 μM LY294002 (LY) before stimulation with Ang II (100 nM). Both losartan and LY294002 completely blocked Ang II-induced phosphorylation of Akt. ADR, adriamycin; p-Akt(308), phospho-Akt-Thr308; p-Akt(473), phospho-Akt-Ser473. Beta-tubulin served as an internal control for normalization purposes.

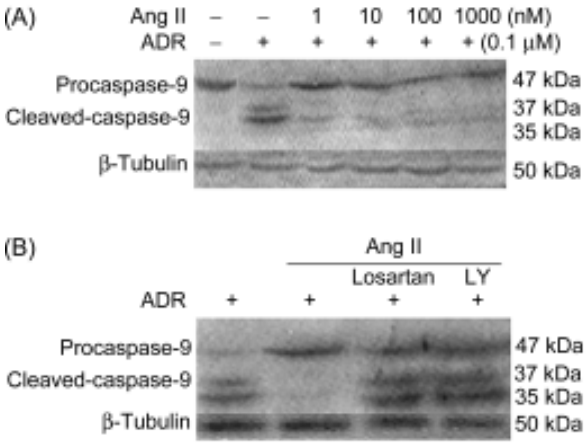

Fig. 4 Angiotensin II (Ang II) inhibits caspase-9 activation in a dose-dependent manner through Ang II type 1 receptor (AT1R) and phosphatidylinositol 3 (PI3)-kinase (A) Dose-response study in which cells were stimulated with the indicated doses of Ang II for 24 h in 0.1 μM adriamycin (ADR) serum-free medium. Pretreatment with 100 nM Ang II significantly suppressed the ADR-induced cleavage of procaspase-9. (B) Role of AT1R and PI3-kinase in the inhibition of caspase-9 activation after Ang II stimulation. The Ang II-induced suppressive effects on the cleavage of procaspase-9 were completely inhibited by pretreatment with losartan or LY294002 (LY). Beta-tubulin served as an internal control for normalization purposes.

human breast tissue express both AT1R and AT2R [23], but that AT1R is overexpressed in breast ductal carcinoma *in situ* [24]. Therefore, Ang II/AT1R could be involved in abnormal breast tissue function [25]. Our studies also show that Ang II significantly promotes MCF-7 cell growth in a dose-dependent and time-dependent manner. Losartan decreased the level of Ang II-induced proliferative effects. However, treatment with losartan alone had no effect on cell viability (data not shown). These results are consistent with the report by Muscella *et al* [5]. Moreover, Ang II could modulate integrin expression in MCF-7 cells through AT1R [26]. However, the role of Ang II on ADR-induced apoptosis in breast cancer is not clear.

We first revealed that Ang II protects MCF-7 cells from ADR-induced apoptosis in a dose-dependent manner by Hoechst 33342/PI staining and nuclear DNA fragmentation analysis. These results were further confirmed by flow cytometry analysis using PI/annexin V. This finding is the first indication of an anti-apoptotic role for Ang II in breast cancer cells. Recently, reports have shown that Ang II stimulates the growth of tumor cells through AT1R [9–12]. In this study, we clearly showed that Ang II plays an anti-apoptotic role in MCF-7 cells and that AT1R, but not AT2R, is the receptor that mediates this mechanism.

We next investigated apoptosis-related signaling pathways and PI3-kinase, previously reported to be down-

stream of AT1R [19]. Ang II is reported to prevent apoptosis in microvascular endothelial cells through the PI3-kinase pathway [27]. In this study, Hoechst 33342/PI staining, nuclear DNA fragmentation analysis, and flow cytometric analysis revealed that 50 μM PI3-kinase inhibitor completely reversed the anti-apoptotic effect of Ang II in MCF-7 cells. Akt has been identified as a major downstream target of PI3-kinase, involved in Ang II-induced proliferation in rat aortic smooth muscle cells [28]. So we used Western blot analyses to determine the phosphorylation status of Thr308 and Ser473. Ang II increased phospho-Akt-Thr308 and phospho-Akt-Ser473 in MCF-7 cells in a dose-dependent and time-dependent manner. These results indicated that the PI3-kinase/Akt pathway has an essential role in anti-apoptosis signaling by Ang II. Because the PI3-kinase/Akt pathway in breast cancer cells has also been shown to play a critical role in chemoresistance [29,30], our results might further support a general concept that this pathway is indispensable for protection against apoptosis.

We further studied the pathways leading to Akt phosphorylation in Ang II/AT1/PI3-kinase signaling. A previous report indicated that PI3-kinase is required for Ang II-induced Akt phosphorylation in vascular smooth muscle cells [15]. In MCF-7 cells, our study showed that losartan and LY294002 significantly inhibit Akt phosphorylation. These data suggest that AT1/PI3-kinase signaling is the mechanism for Akt activation by Ang II.

The intracellular machinery responsible for apoptosis depends on a family of caspases. Caspase-9 is a key caspase involved in ADR-induced apoptosis [31]. Another mechanism whereby Akt functions to promote survival is through the phosphorylation and inactivation of procaspase-9, because Akt has been found to phosphorylate procaspase-9 and thereby inhibit its protease activity [20]. Therefore, we focused on the effect of Ang II on procaspase-9. In our studies, Ang II pretreatment suppressed the ADR-induced cleavage of procaspase-9, and this inhibition was suppressed in the presence of losartan or LY294002. Thus, it seems that AT1R and PI3-kinase/Akt pathways could be essential for Ang II to transmit its protective signal against ADR-induced apoptosis.

In summary, our results show that Ang II is a prominent anti-apoptotic molecule in breast cancer MCF-7 cells and the underlying molecular mechanism for this effect involves AT1R and PI3-kinase/Akt. We further uncovered that a novel signaling pathways responsible for Ang II-induced anti-apoptosis was inhibition of caspase-9 activation. Moreover, De Paepe *et al* [24] proved that normal mammary epithelial HMec cells showed little effect when treated with increasing concentrations of Ang II and losartan. Thus, the present observations suggest that clinical benefits in treating patients with breast cancer could be obtained with appropriate combinations of novel AT1R inhibitors and conventional chemotherapeutic drugs.

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