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Regulation of autophagy and apoptosis in response to angiotensin II in HL-1 cardiomyocytes

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ABSTRACT

Background: Autophagy and apoptosis are two important regulators of cell survival, and are often observed simultaneously in response to noxious stimuli. Anoxia is a known stimulus for autophagy and apoptosis, and angiotensin (Ang) II is a major mediator of anoxic injury. However, specific responses to anoxia and Ang II in terms of occurrence of autophagy and apoptosis have still not been delineated.
Methods and results: We observed that autophagy (measured as LC3 staining, and Beclin-1 and p62 Western blotting) was an early response and apoptosis (measured as TUNEL staining, and Annexin V and Smac/Diablo Western blotting) became dominant as the duration of anoxia was prolonged. Autophagy also occurred quickly in response to low concentrations of Ang II. When exposed to high concentrations of Ang II, a significant number of cells developed apoptosis, while autophagy response decreased. Ang II-mediated apoptosis was blocked by Ang II type 1 receptor (AT1R) blocker losartan as well as by the AT2R blocker PD123319. Ang II-induced autophagy was blocked by losartan, but not by PD123319.
Conclusion: Exposure to Ang II, a mediator of anoxic injury, initiates a rapid autophagy response, perhaps in an attempt to protect tissues from the impending noxious effects. However, when anoxia (and thereby release of Ang II) is prolonged, the process of apoptosis dominates. These processes will determine the outcome of cardiomyocyte well-being in states of hypoxia.

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1. Introduction

The occurrence of apoptosis and autophagy has been described in several disease states, including, atherosclerosis, hypertension, myocardial ischemia and certain tumors [1,2]. Apoptosis, also known as programmed cell death, is a naturally occurring homeostatic process in the body that regulates cell death. Autophagy is another homeostatic process involved in the degradation and recycling of the unnecessary and dysfunctional cellular components [3]. Autophagy has complex relationships with apoptosis. Generally, autophagy is believed to protect the cells from impending death, and apoptosis suppresses cell autophagy [1,3]. However, under certain specific circumstances, autophagy itself can cause cell death, a phenomenon known as autophagy-mediated apoptosis [1].

Many stimuli can simultaneously cause cell apoptosis and autophagy. Chen et al. showed that hypoxia could cause autophagy in human trophoblasts, and blocking autophagy by bafilomycin enhanced apoptosis [4]. Song et al. found that autophagy resulting from hypoxia protected tumor cells from apoptosis [5]. Other studies have shown that persistent hypoxia results in initiation of apoptosis [6–8]. Mazure et al. reported that hypoxia (~3–0.1% oxygen) is able to induce autophagy and cell survival, however, severe hypoxic conditions or anoxia (0.1% oxygen) often causes cell death [9]. Anoxia results in the release of angiotensin II (Ang II) which has been shown to induce autophagy as well as apoptosis [10–14]. We posited that the tissue response following exposure to Ang II and anoxia may be determined by the duration and intensity of these stimuli. This study addresses this question.

2. Materials and methods

2.1. Cell culture and study protocol

HL-1 cardiomyocytes were cultured as reported previously [13]. Cells were exposed to different concentrations of Ang II (10^{-8}–

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10^{-5} M) for 12 h. Based on preliminary data on cell apoptosis and autophagy, 10^{-8} M concentration of Ang II was selected to study time-response of autophagy, and 10^{-6} M concentration was selected to study time-response of apoptosis (exposure time: 3–24 h). In other experiments, cells were pretreated with the Ang II type 1 receptor (AT1R) blocker losartan (5 μM) or AT2R blocker PD123319 (5 μM) for 12 h before exposure to Ang II for the next 12 h.

2.2. Exposure to anoxia

HL-1 cells were plated in 6-well or 24-well plates. When cells reached 70% confluency, they were kept in a controlled atmosphere chamber (Plas-Labs, Lansing, MI) under 100% N_2 (referred as anoxia) and 37 °C for 1–24 h. Cells were then taken out of the anoxia chamber and placed in general cell culture incubator for additional 1 h at 37 °C (reoxygenation).

2.3. TUNEL staining for detection of apoptosis

HL-1 cells were grown on 10 mm round coverslips and treated as per above-mentioned protocols. Apoptosis was detected using a DeadEnd™ Fluorometric TUNEL kit (Promega Corporation, Madison, WI), as per manufacturer's instructions.

2.4. Immunostaining for detection of autophagy

The methodology has been described earlier in detail [15]. In brief, HL-1 cells were grown on 10 mm round coverslips, and fixed in 4% formaldehyde and treated with 0.2% Triton X-100. After washing with PBS, the cells were incubated with rabbit anti-mouse LC-3 antibody (Abcam, Cambridge, MA) for 1 h at room temperature. The cells were then washed 3 times and incubated with anti-rabbit secondary antibody (Abcam) for 30 min. The cells were then washed, stained with DAPI, and then imaged with a fluorescence microscope.

For double staining of autophagy and apoptosis, cells were first stained with LC-3 antibody, and then for apoptosis.

2.5. Western blotting for detection of apoptosis and autophagy

Protein was extracted from different sets of HL-1 cardiomyocytes, separated by 12% SDS–PAGE gel, and transferred to PVDF membranes. The membranes were blocked with 5% BSA in TBS-T, and then incubated with rabbit anti-mouse Annexin V, Smac/DIABLO, Beclin-1 or p62 antibodies (Abcam), at 4 °C overnight. The blots were then incubated with HRP-conjugated duck anti-rabbit second antibody (Abcam) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence.

2.6. Statistical analysis

Statistical analysis was performed with SPSS 11.5 software. Data are presented as mean ± SD from at least 4 independent experiments. Univariate comparisons of means were assessed by a one-way ANOVA with a Newman–Student–Keul t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Autophagy and apoptosis in HL-1 cells exposed to anoxia

First, we studied the effect of varying duration of anoxia (1–24 h). As shown in Fig. 1, autophagy signals (LC-3 staining and Beclin-1 expression determined by Western blotting) increased soon after initiation of anoxia, and then began to decrease as the duration of anoxia was increased. Of note, the autophagy signals remained higher than baseline even when the duration of anoxia was as long as 24 h. In contrast to the autophagy that occurred early after exposure to anoxia, apoptosis signals (TUNEL staining and Annexin V expression determined by Western blotting) began to appear late (at 3 h after anoxia) and then continued to increase as the duration of anoxia was increased. Of note, anoxia was followed by 1 h of reoxygenation in all these experiments.

LC-3 and Beclin-1 are two independent markers of autophagy [16]. Ablation of Beclin-1 has been observed to enhance cell apoptosis under hypoxia conditions [17]. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades [18]. Annexin V is used to specifically target and identify apoptotic cells [19].

3.2. Autophagy and apoptosis in HL-1 cells exposed to Ang II

We exposed HL-1 cells to 10^{-8}–10^{-5} M Ang II for 12 h. At baseline (without exposure to Ang II, or Control), LC-3 staining and TUNEL positivity both were very low in these cells (Fig. 2A and B). After exposure to the smallest concentration of Ang II (10^{-8} M), LC-3 expression increased, and quickly reached the peak value at 10^{-7} M Ang II, and then began to decrease as the concentration of Ang II was increased, but was still higher than at baseline. Ang II at 10^{-8} and 10^{-7} M concentrations had no significant effect on cell apoptosis, but at high concentrations (10^{-6} and 10^{-5} M), apoptosis became very prominent (Fig. 2A and B). The staining data were confirmed by Western-blot assay, which showed that the patterns of autophagy (Beclin-1) and apoptosis (Annexin V) in response to different concentrations of Ang II were similar to the changes in LC3 and TUNEL staining data (Fig. 2C).

Next, we measured apoptosis in HL-1 cardiomyocytes exposed to Ang II 10^{-6} M for 3–24 h to study time-response. As shown in Fig. 3A, cell apoptosis increased in a time-dependent manner. The results of Annexin V and Smac/Diablo [Second mitochondria-derived activator of caspase also known as DIABLO (Direct IAP-Binding Protein with Low Pi)] expression (Western-blotting), two different markers of apoptosis, were similar to TUNEL staining (Fig. 3A). Overexpression of Smac/DIABLO is a marker of increased sensitivity of the cell to apoptic stimuli [20].

Next, we measured autophagy in cells following exposure to a low concentration of Ang II (10^{-8} M) for varying periods (3–24 h). As shown in Fig. 3B, the expression of autophagy (measured as LC-3 staining and Beclin-1 Western blotting) was evident very early and increased in a time-dependent manner. We also measured p62 which serves as a useful marker for the induction of autophagy, and clearance of protein aggregates [21]. p62 expression decreased as the duration of exposure to Ang II was increased.

3.3. Role of AT1R and AT2R in Ang II-induced autophagy and apoptosis

Previous studies have shown that Ang II-induced apoptosis is mediated by type 1 receptor (AT1R) activation [12]. Some studies have suggested that AT2R activation also participates in the development of apoptosis [22–24]. To delineate the role of AT1R and AT2R in apoptosis in the present study, cells were pretreated with the AT1R blocker losartan or AT2R blocker PD123319 before treatment with Ang II. We observed that Ang II-induced apoptosis was inhibited by pretreatment of cells with losartan or PD123319 (Fig. 4A), which suggests that Ang II-induced apoptosis of HL-1 cells is mediated via activation of both AT1R and AT2R.

In support of the reports that AT1R activation participates in the development of autophagy [10], we observed that Ang II-induced autophagy signals (LC3 and Beclin-1) were blocked by losartan,

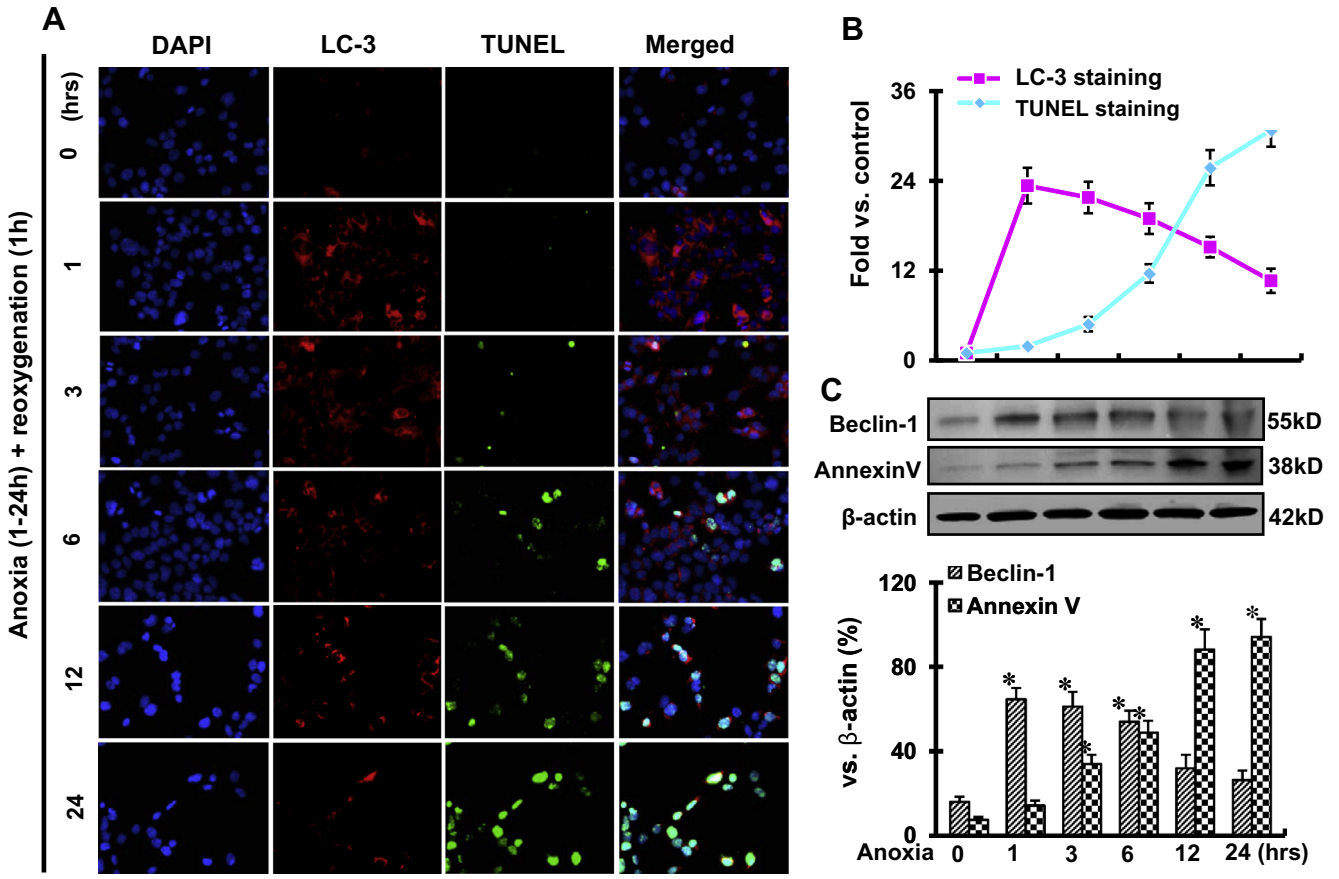

Fig. 1. Autophagy and apoptosis in HL-1 cells exposed to 1–24 h of anoxia and 1 h of reoxygenation. (A) LC3 immunopositivity (index of autophagy) increases early after exposure of cells to anoxia (as early as 1 h). TUNEL staining (apoptosis signal) appears to increase at 3–6 h and becomes prominent with longer duration of anoxia. (B) Quantification of fluorescence density of LC3 and TUNEL staining over time. (C) Western-blotting assay shows expression of Beclin-1 and Annexin V after exposure of cells to anoxia. Results are parallel to the data on LC-3 positivity and TUNEL staining. Data are representative of 4 independent experiments. Graphs show mean (±SD) values. $^{*}P < 0.05$ vs. control.

but not by PD123319 (Fig. 4B). These observations suggest that Ang II-induced autophagy response is dependent on AT1R activation.

4. Discussion

In this study, we investigated concentration- and time-dependent development of autophagy and apoptosis in HL-1 cardiomyocytes following exposure to Ang II, a major mediator of anoxic tissue injury. We found that autophagy and apoptosis responses to Ang II are dependent on the concentration as well as duration of exposure of cells to Ang II. Autophagy occurs early and at low concentrations whereas apoptosis occurs late and at higher concentrations. The autophagy response declines at higher concentrations of Ang II, but still continues to be higher than the baseline value. These observations indicate that cardiomyocytes respond by developing autophagy very early after exposure to mediators of tissue injury. As the injurious stimuli continue to bathe the cardiomyocytes, these cells exhaust their endogenous defenses and begin to exhibit apoptosis. These observations expand on our knowledge of the cardiomyocyte response to injurious stimuli.

Autophagy is a self-degradative process that is involved in removing and recycling damaged proteins, maintaining intracellular homeostasis, and maintaining cell survival [25]. Many studies have suggested that this process is increased in the ischemic myocardium as well as in the failing heart [1,25].

Cao et al. [26] showed that autophagy develops in the ischemic heart. The present studies clearly demonstrate that a short period (as little as 1 h) of exposure to anoxia leads to the development of autophagy. Ang II in very small concentrations, i.e., 10^{-8} M, which is likely to be present in the heart early during ischemia, initiates autophagy. As anoxia persists and Ang II concentrations begin to rise, apoptosis becomes the dominant phenomenon. However, autophagy is still higher than at baseline.

Release of Ang II in the ischemic tissue has traditionally been thought to be related to the development of apoptosis and necrosis of cardiomyocytes. Both AT1R and AT2R have been incriminated in the development of apoptosis [22–24]. Recent reports suggest that AT1R activation may also be a mediator of autophagy [10]. In keeping with these observations, we found that Ang II-mediated apoptosis was blocked by the AT1R inhibitor losartan as well as the AT2R inhibitor PD123319. However, autophagy was blocked only by losartan suggesting that AT2R activation does not participate in the development of autophagy.

Autophagy has a complex interaction with apoptosis. It can inhibit apoptosis, and sometime itself can cause cell apoptosis (known as autophagy-mediated apoptosis). On the other hand, cell apoptosis is known to inhibit the genesis of autophagy [1,3]. We observed that autophagy was still higher than at baseline when anoxia was continued, or when the concentrations of Ang II were high. It is probable that autophagy at this time point adds to cell injury and contributes to the expansion or extension of tissue injury.

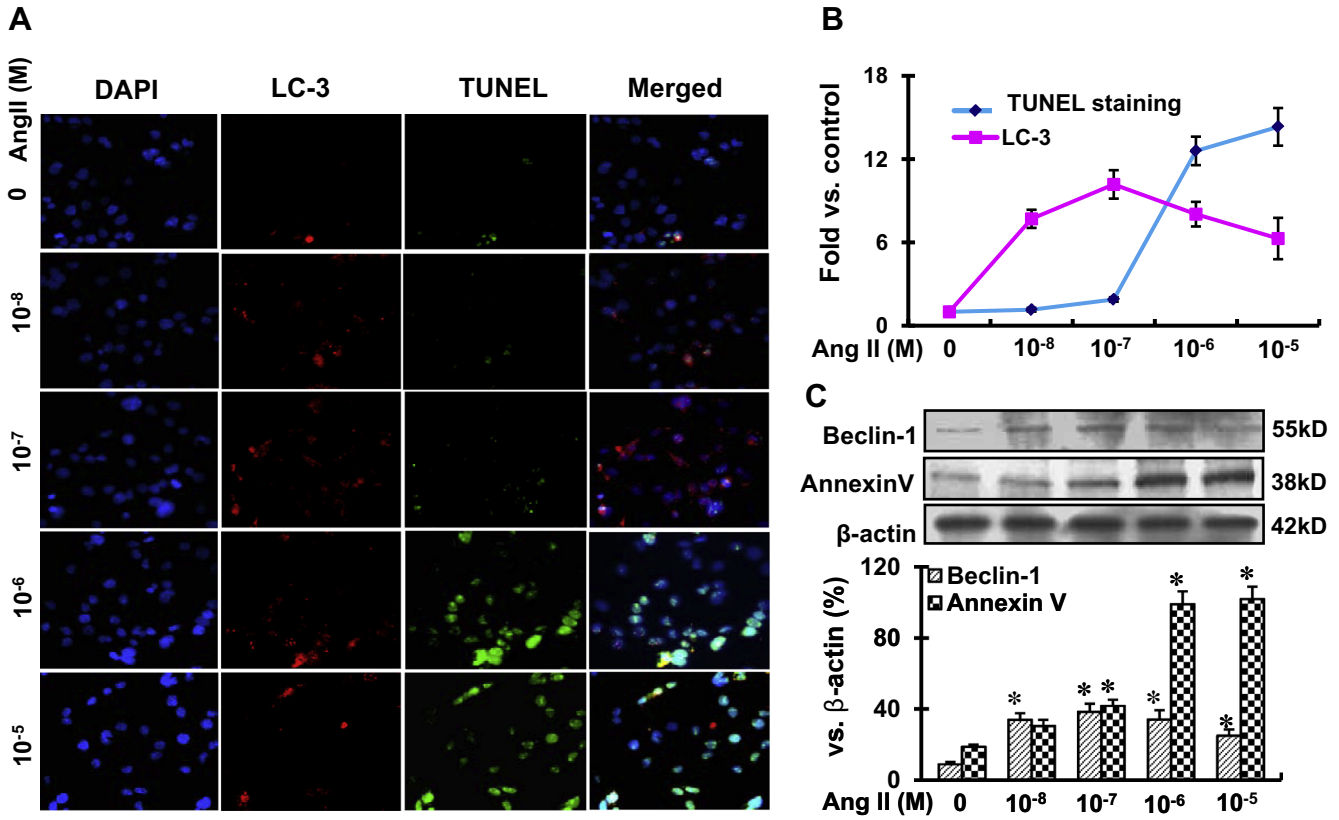

Fig. 2. Autophagy and apoptosis in HL-1 cells exposed to different concentrations of Ang II (10^{-8}–10^{-5} M) for 12 h. (A) LC-3 immunostaining increases in response to very small concentrations of Ang II. On the other hand, TUNEL staining suggests that apoptosis occurs in response of high concentration of Ang II. Thus at high concentrations, apoptosis dominates but autophagy is still higher than baseline. (B) Quantification of fluorescence density of LC3 and TUNEL staining in HL-1 cells in relation to concentrations of Ang II. (C) Western-blotting assay for Beclin-1 and Annexin V confirms the results of LC3 and TUNEL staining. Data are representative of 4 independent experiments. Graphs show mean (±SD) values. $^{*}P < 0.05$ vs. control.

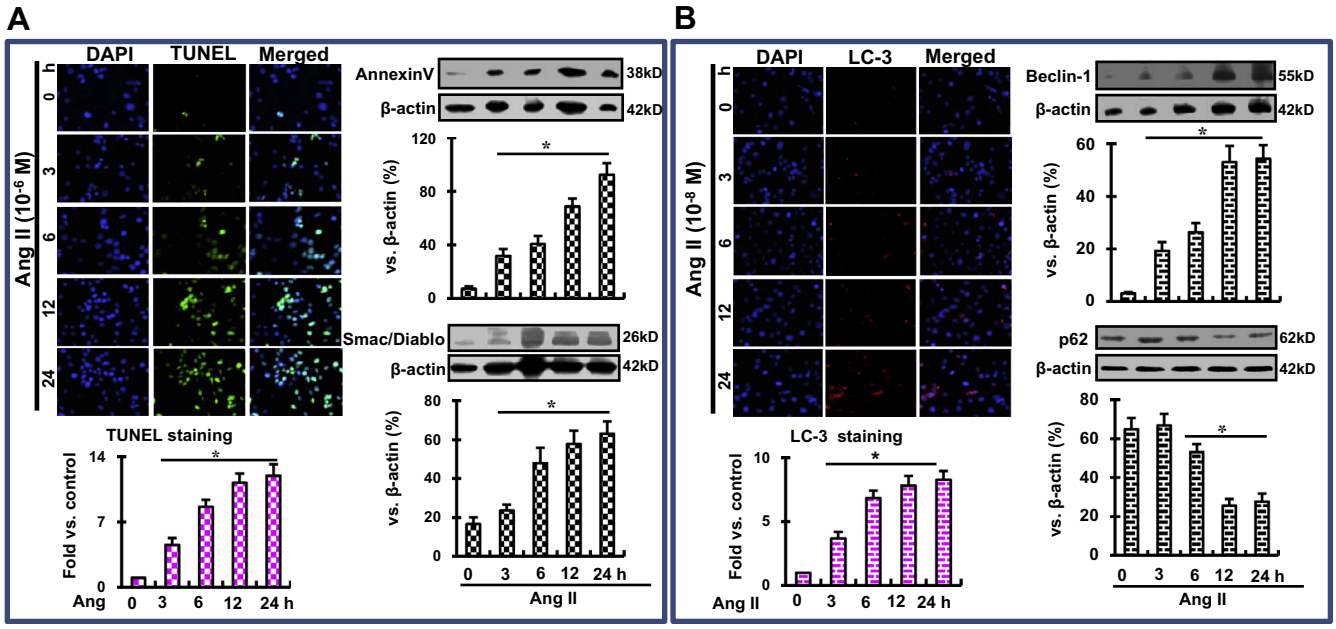

Fig. 3. Time-response of apoptosis and autophagy in HL-1 cells after exposure to Ang II for 3–24 h. (A) TUNEL staining shows a time-dependent increase in apoptosis in HL-1 cells exposed to Ang II (10^{-6} M). Western-blotting assay shows similar increase in apoptosis with time (Annexin V and Smac/Diablo expression) in cells exposed to Ang II. (B) LC3 immunostaining increases very early and continues to increase in a time-dependent manner after exposure to Ang II (10^{-8} M). Western-blotting assay for Beclin-1 confirms the results of LC-3 staining. Note that p62 expression begins to decrease at 6 h following exposure to Ang II. Data are representative of 4 independent experiments. Graphs show mean (±SD) values. $^{*}P < 0.05$ vs. control.

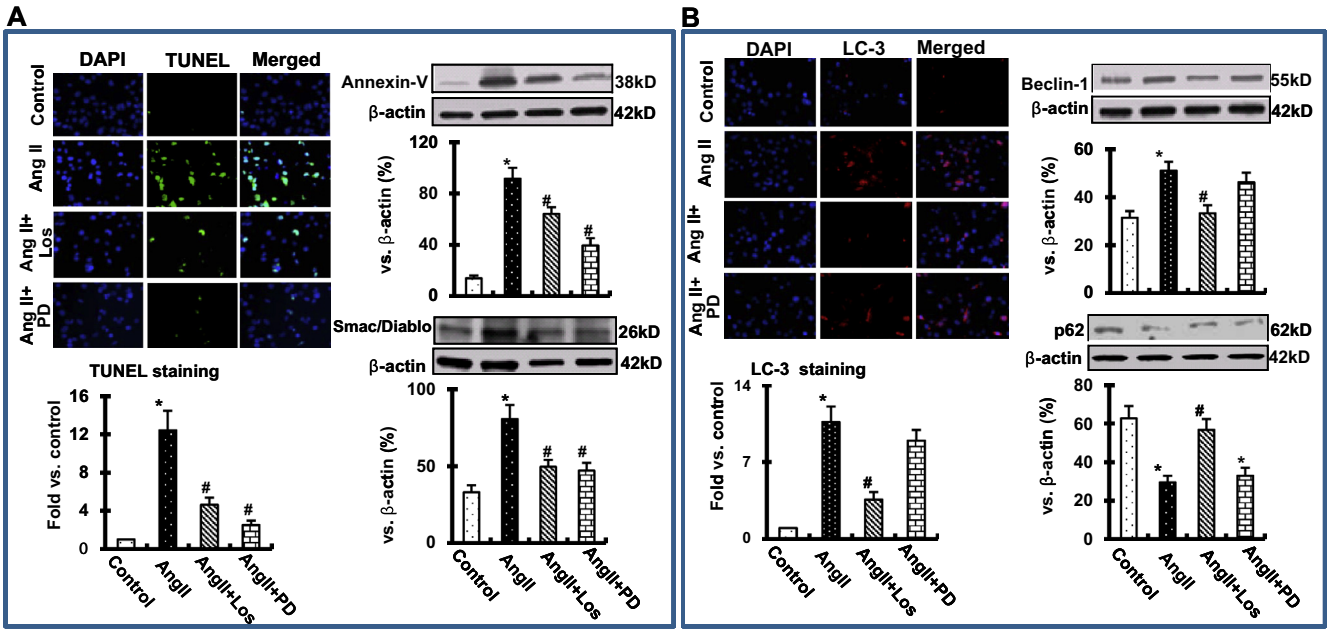

Fig. 4. Effects of the AT1R blocker losartan (Los) and the AT2R blocker PD123319 (PD) on Ang II-induced apoptosis and autophagy of HL-1 cells. (A) TUNEL staining shows reduction in apoptosis in cells pretreated with Los or with PD. Western-blotting assay shows Annexin V and Smac/Diablo expression is also reduced in cells pretreated with Los or PD. (B) Autophagy marker LC-3 is reduced in cells pretreated with Los, but not with PD. Western-blotting assay shows Beclin-1 expression follows the same pattern as LC-3. Reduced expression of p62 following Ang II is ameliorated by pretreatment of cells with Los but not PD. Data are representative of 4 independent experiments. Graphs show mean (±SD) values. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. treatment with Ang II.

The description of early occurrence of autophagy in response to anoxia and Ang II described in this study is novel, and may have relevance in the treatment of ischemic disease states. Studies from our laboratory showed that small concentrations of noxious stimuli, such as Ang II, may have salutary effects by initiating tissue protective mechanisms [27]. Early studies with the use of autophagy enhancing approach have also shown promising results [28], and support the concepts presented here. Agents that modulate Ang II activity may also modify the process of autophagy and apoptosis, and result in amelioration of hypoxic tissue injury.

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