

WITHDRAWN: The Study on Angiotensin II Induced-ferroptosis in Vascular Endothelial Cells

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Abstract

Background: To verify the effect of Angiotensin II on ferroptosis in vascular endothelial cells and clarify the related mechanism.

Methods: HUVECs were evaluated for p53, P21, ALOX12, VEGF, MDA, GSH. Molecular marker impact upon AngII-induced ferroptosis was evaluated with students' t-test one-way analysis of variance (ANOVA).

Results: As the concentration of Ang II increased the level of ALOX12, P53, GSH and MDA increased in HUVECs. The expression of VEGFA in HUVECs is negatively correlated with dose of Ang II. Incubation of HUVECs in AngII and valsartan for 48hr reduces ALOX12, P21, GSH and MDA. Compared with the single AngII group, ALOX12, P21, GSH and MDA in valsartan group was decreased significantly $p=0.000$. In pifithrin- α hydrobromide-treated, ALOX12, P21, GSH and MDA was reduced significantly, as compared to valsartan group $p=0.000$. The most larger reduction in ALOX12, P21, GSH and MDA was pifithrin - α hydrobromide combined with valsartan group. In contrast, the expression of VEGFA increased significantly after HUVECs were treated with pifithrin - α hydrobromide and valsartan $p=0.000$.

Conclusions: AngII can induce ferroptosis of vascular endothelial cells in a dose-dependent manner. The mechanism of AngII-induced ferroptosis may be regulated through the signal axis of ATR1,2-p53-ALOX12.

1. Background

Ferroptosis is a new form of programmed cell death that is different from apoptosis, necroptosis and autophagy at both the morphological and biochemical levels and has characteristic accumulation of reactive oxygen species (ROS) resulting from iron accumulation and lipid peroxidation. Recent studies have shown that ferroptosis plays an important regulatory role in the occurrence and development of many diseases and has become the focus and hotspot of research on the treatment and prognosis improvement of related diseases. [1–4]

The endothelium lies in between the circulating blood and vascular smooth muscle cells, which are responsible for peripheral resistance. It may be easily damaged and endothelial dysfunction occurs in the pathogenesis of various cardiovascular complications, particularly in hypertension. Endothelial dysfunction contributes to an increase in large arterial stiffness in patients with isolated systolic hypertension, resulting in impaired vascular elasticity and compliance, and subsequent arterial hypertension. Evidence from previous studies suggest that angiotensin II (AngII), a peptide of the rennin angiotensin system, is considered a proinflammatory mediator in hypertension through mechanisms involving production of ROS. [5, 6] However, it is unclear whether AngII can induce ferroptosis in vascular endothelial cells and the underlying mechanism remains elusive. Therefore, in the present study, we investigated the effect of AngII on vascular endothelial ferroptosis and the possible mechanism using human umbilical vein endothelial cells (HUVECs).

2. Materials And Methods

2.1 Cell culture

For all cell culture studies, primary HUVECs were used. HUVECs were cultured in 1.5% gelatin-coated cell culture dishes, and cells at passages 4–8 were used in the experiments. [7] At first, the cells were divided into the following six groups: control group; 0.1uM AngII ; 1uM AngII ; 10uM AngII ; 100uM AngII ; 1000uM AngII. Different groups were treated with different concentrations of AngII. In a second experiment, cells in all groups were treated with ang II. Then, cells in four groups were treated in different methods respectfully. Valsartan was used in AT1R blocking group. The p53 block group was incubated with pifithrin- α hydrobromide. AT1R and p53 simultaneously blocked group was treated with valsartan and pifithrin- α hydrobromide at the same time.

2.2 Measurement of Glutathione and Malonaldehyde

The concentration of reduced glutathione(GSH) and malonaldehyde(MDA) released by HUVECs was evaluated using an ELISA assay. Briefly, 100 μ L different dilutions of the standard sample were added to the reaction plate and incubated at 37°C for 30 minutes. Secondly, to each well, 100 μ L of test samples was added to the plate after washes and incubated at 37°C for 2 hours. Thereafter, the plates were washed before the addition of 100 μ L horseradish peroxidase-labeled secondary antibody and incubated at 37°C for 30 minutes. Afterward, 50 μ L developer A and 50 μ L developer B were added. The plates were kept in the dark for 15 minutes. Lastly, to each well, 50 μ L stop solution was added into the plate to terminate the reaction. The optical density of the plate was read using an ELISA reader, and the concentration of the test sample was calculated.[8]

2.3 Determination of Arachidonic acid 12-lipoxygenase, Vascular endothelial growth factor, p53, P21

After treatment with the corresponding drugs, HUVECs were lysed in RIPA buffer containing a mixture of protease inhibitors and centrifuged to extract total protein. Protein concentrations were determined using a BCA protein assay kit according to the manufacturer's instructions, and 25 μ g of protein was analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes with transferred proteins were blocked with 5% skim milk for 1 hour and incubated overnight at 4°C with antibodies against the following proteins: Arachidonic acid 12-lipoxygenase (ALOX12), Vascular endothelial growth factor (VEGFA),P53,P21, and GAPDH. The relative intensities of protein bands were analyzed using ImageJ software.[9]

2.4 Statistical analysis

Mean \pm standard deviation (SD) was displayed to show the data. SPSS 22 statistical software package was used to analyze the data. Students' t-test and one-way analysis of variance (ANOVA) were utilized for the contrast among different groups. Multiple regression assessed associations between ALOX12, P53, P21, VEGFA and vascular endothelial ferroptosis. P values of less than 0.05 were considered statistically significant.

3. Results

3.1 The relation between concentration of Ang II and vascular endothelial ferroptosis:

As the concentration of Ang II (0,0.1,1,10,100, and 1000uM for 48 h) increased the level of ALOX12, P53,GSH and MDA increased in HUVECs. The expression of VEGFA in HUVECs is negatively correlated with dose of Ang II. (Fig. 1,2, Table 1,2)

3.2. Impacts of AT1R antagonist, P53 inhibitor on vascular endothelial ferroptosis

Incubation of HUVECs in AngII and valsartan for 48hr reduces ALOX12, P21, GSH and MDA. Compared with the single AngII group, ALOX12, P21, GSH and MDA in valsartan group was decreased significantly. In pifithrin- α hydrobromide-treated, ALOX12, P21, GSH and MDA was reduced significantly, as compared to valsartan group. The most larger reduction in ALOX12, P21,GSH and MDA was pifithrin - α hydrobromide combined with valsartan group. In contrast, the expression of VEGFA increased significantly after HUVECs were treated with pifithrin - α hydrobromide and valsartan. (Fig. 2, Table 2)

4. Discussion

In the present study, by stimulating HUVECs with angiotensin II, the production of MAD and GSH was significantly promoted. These data indicated that level of MAD and GSH in HUVECs was greatly elevated by angiotensin II, which was a factor that induced HUVEC ferroptosis. The result in our study demonstrated that angiotensin II increased HUVECs ferroptosis in a dose-dependent manner.

Studies have shown that ferroptosis is closely related to the pathophysiological processes of more and more diseases. The discovery of ferroptosis has opened up a new platform in the field of disease research, and its clinical significance in the occurrence, development, and treatment of diseases has gradually emerged. With the deepening of the research, ferroptosis has been found in the pathophysiological processes and diseases, and it provides a new method for treating these diseases.[1–4, 10, 11]

Ferroptosis is a form of lipid peroxidation-induced cell death that can be regulated in many ways, from altering the activity of antioxidant enzymes to the level of transcription factors. The p53 is 'the guardian of the genome' that participates in the control of cell survival and division under various stresses. Beyond its effects on apoptosis, autophagy, and cell cycle, p53 also regulates ferroptosis either through a transcriptional or posttranslational mechanism. p53-mediated activation of p21 was reported to promote the conservation of glutathione.[1–4, 10, 11]

ALOX12 is critical for p53-mediated ferroptosis. [12, 13]Inactivation of ALOX12 can reduce p53-mediated ferroptosis caused by active oxygen stress. ALOX12 was shown to be related to the ferroptosis independent of ACSL4 [14]. The ALOX12 gene resides on human chromosome 17p13.1.The ALOX12 protein specifically catalyzes the addition of molecular oxygen to arachidonic acid to produce a

biologically active lipid medium, such as 12-hydroxyeicosatetraenoic acid(12-HETE) [12, 13]. As an important lipoxygenase, ALOX12 plays a significant role in biological processes. Its metabolites are not only necessary for normal biological processes but also the basis for many diseases. A large body of evidence indicates that methylation modification of ALOX12 and its genetic variation and oxidative stress in the environment affect the expression and function of ALOX12. Because ALOX12 has the function of regulating platelet aggregation, cell migration, and tumor cell proliferation, it is mainly involved in the development of diseases such as thrombosis, atherosclerosis, and cancer. Besides, ALOX12 is involved in the regulation of inflammation and apoptosis. ALOX12 plays an important role in the occurrence and development of diseases. ALOX12 R261Q is associated with the risk of essential hypertension in the Spanish population. ALOX12 expression was reduced in carotid atherosclerosis. Down-regulation of ALOX12 blocks a response caused by vascular endothelial contraction. In the arteries of mice, ALOX12 metabolizes arachidonic acid to produce thromboxane receptor antagonists, which effectively stretch blood vessels [15]. In addition, the increased expression of ALOX12 in the vascular smooth muscle of hypertensive rats can increase the expression level of Ang II subtype 1 receptor [16], and a study has shown that the increased expression of Ang II receptor is significantly correlated with the severity of pregnancy-induced hypertension [17]. Excess aldosterone can upregulate the expression of ALOX12 and ALOX15 in human vascular smooth muscle, thereby increasing the risk of LDL oxidation and atherosclerosis [18]. In this study, Ang II was used to treat HUVECs; it increased p53 and ALOX12 of HUVECs in dose-dependent manners. The level of MAD, GSH, p53 and ALOX12 in HUVECs was positively correlated to dose of Ang II. Furthermore, our results showed that MAD, GSH and ALOX12 decreased after blocking AT1R and/or p53. ALOX12, P21, GSH and MDA was partially attenuated by the AT1R antagonist. Moreover, the level of ALOX12, P21, GSH and MDA was reduced further in HUVECs treated with both p53 inhibitor and AT1R blocker, indicating the involvement of both AT1R and p53 in the Ang II-induced HUVECs ferroptosis. A lot of literature reports that AT2R is one of the functional receptors of Angiotensin II, which has been proven to mediate the progress of apoptosis.[8] In our current study, the inhibitory effect of p53 was stronger than that of AT1R, indicating that AT2R may also be involved in this process.

The VEGF family exerts their biological functions through the interaction with transmembrane receptors such as tyrosine kinase receptors VEGFR1 and VEGFR2. The ligands which specifically bind to VEGFR1 are VEGF-A, -B and PlGF while those bind to VEGFR2 are VEGF-A, -C, -D and -E4,5. Binding of VEGFs to VEGF receptor-1 and -2 triggers downstream signaling pathways resulted in EC proliferation, migration, invasion and high vascular permeability. Pfaff et al. have reported that augmentation of p53 expression could decrease the levels of VEGFA in an ischemia-induced angiogenesis and arteriogenesis mouse model. However, the roles of p53 in the regulation of VEGFA have always been controversial. Quite a few studies have reported that p53 inhibits the expression of VEGFA. Other studies have reported that the expression of p53 is positively related to the expression of VEGFA.[19–24] Chen et al. reported that p53 suppressed expression of VEGFA through miR-1249.[25] The ALOX12 product 12S-HETE also increased the release of VEGF, whereas ALOX12 inhibitors had the opposite effect, suggesting that 12S-HETE is also an endogenous regulator of VEGF secretion by these cells. Overexpression of ALOX12 had a similar effect. [26]These controversial results may be due to different cells. In our study, we have founded that

VEGF is negatively correlated with dose of Ang II in HUVECs. However, there was a positive correlation between p53 and AngII. The expression of VEGFA increased after AT1R and/or p53 was inhibited. These results indicate that over high concentration of AngII can promote the production of p53, inhibit the expression of VEGFA, and induce ferroptosis of vascular endothelial cells.

Our study is limited by the fact that we did not observe the effect of AT2R and ALOX12 to be blocked in HUVECs directly. Another limitation is that there are few indicators to evaluate ferroptosis in our study. In spite of these limitations, the data from our experiments can still reveal many problems.

5. Conclusion

In conclusion, our present results suggest that AngII can induce ferroptosis of vascular endothelial cells in a dose-dependent manner. The mechanism of AngII-induced HUVECs ferroptosis may be regulated through the signal axis of ATR1,2-p53-ALOX12.

Declarations

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

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Tables

Tab 1 Effects of different concentrations of AngII on MAD, GSH, ALOX12, VEGFA and P53

AngII (uM)	0	0.1	1.0	10	100	1000	<i>F</i>	<i>p</i>
VEGFA	1±0.235	0.81±0.07	0.72±0.026	0.623±0.05	0.223±0.06	0.293±0.075	75.816	0.000
ALOX12	0.1±0.01903	0.1±0.02	0.103±0.022	0.101±0.033	0.754 ±0.015	0.677±0.017	30.396	0.000
P53	0.1±0.114	0.113±0.042	0.122±0.014	0.285±0.015	0.415±0.082	0.374±0.014	75.117	0.000
MDA	0.297±0.067	0.305±0.047	0.345±0.091	0.412±0.075	0.987±0.015	1.01±0.079	75.548	0.000
GSH	2.969±0.441	3.03±0.354	3.275±0.299	3.137±0.297	8.7±0.16	8.9±0.129	25.399	0.000
<i>F</i>	3.123							
<i>P</i>	0.049							

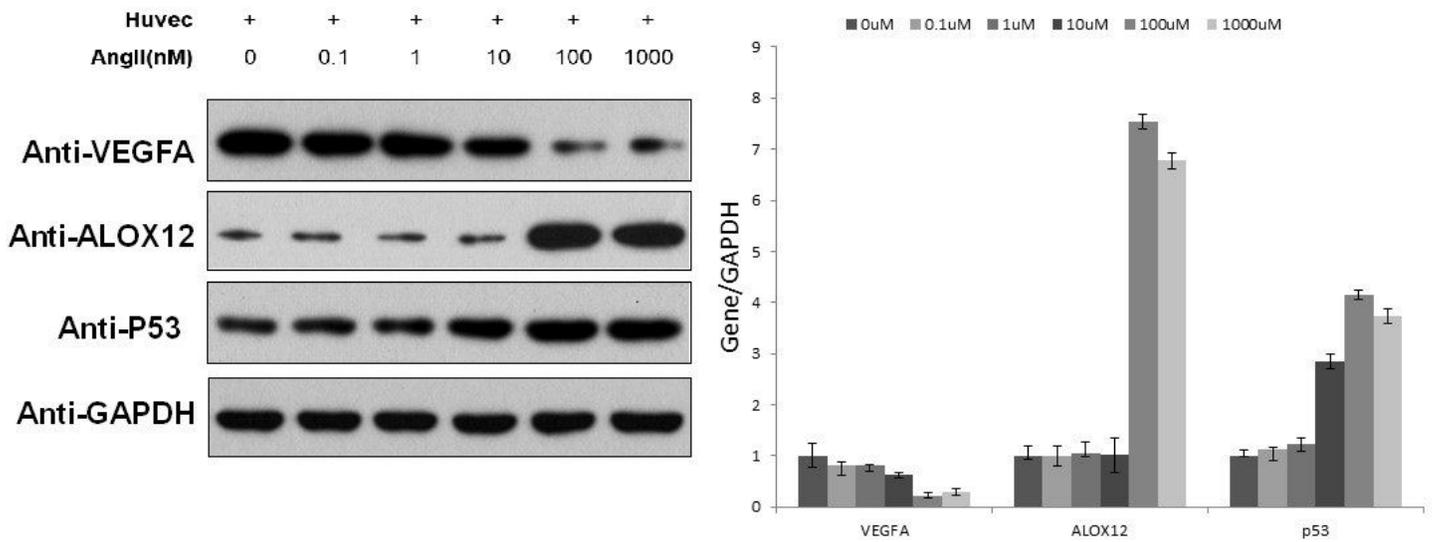
Tab 2 Multiple regression analysis to investigate the relationship of Ang II and ALOX12, P53, VEGFA,MDA,GSH in vascular endothelial cells

	ALOX12	P53	VEGFA	MDA	GSH
Standardized β	0.696	0.364	-0.71	0.98	0.33
p value	0.002	0.045	0.008	0.001	0.036

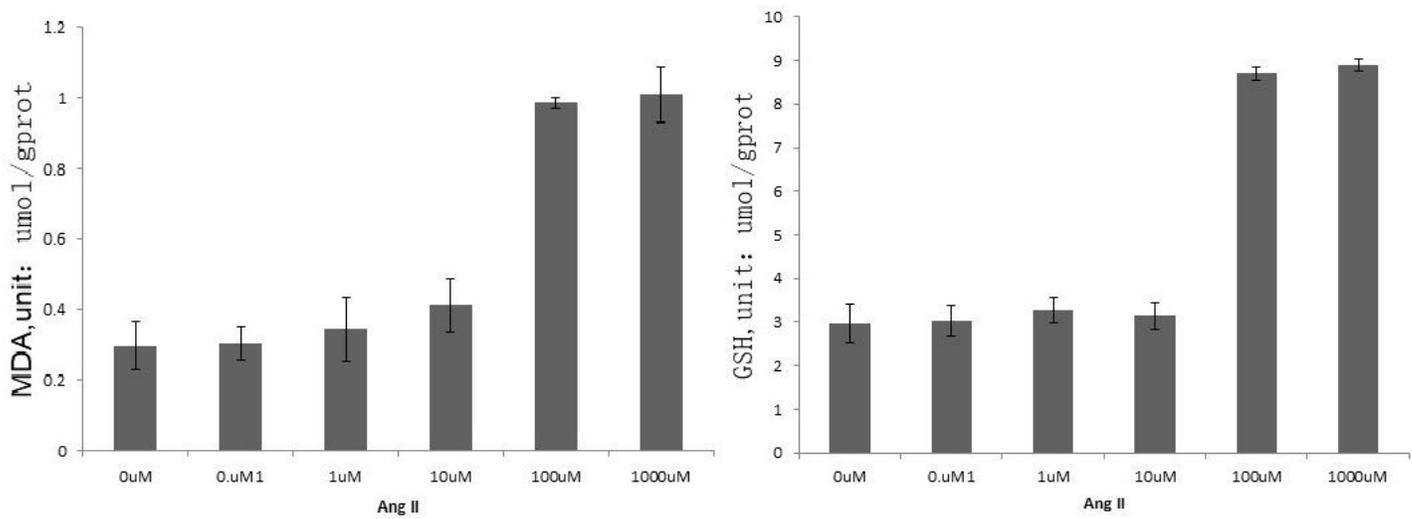
Tab 3 Effects of Losartan , Pofithrin-X on MAD, GSH, ALOX12, VEGFA and P53

	P21	VEGFA	ALOX12	MDA	GSH
Ang II	0.813 \pm 0.057	0.492 \pm 0.019	0.938 \pm 0.051	0.987 \pm 0.015	8.7 \pm 0.16
AngII+Pofithrin-X	0.788 \pm 0.033	0.501 \pm 0.021	0.445 \pm 0.019	0.56 \pm 0.012	4.158 \pm 0.091
AngII+Losartan	0.868 \pm 0.025	0.595 \pm 0.031	0.52 \pm 0.02	0.714 \pm 0.08	5.475 \pm 0.052
AngII+Losartan+Pofithrin-X	0.637 \pm 0.048	0.602 \pm 0.049	0.297 \pm 0.028	0.394 \pm 0.022	2.236 \pm 0.135
F	32.37	18.112	11.911	43.09	52.667
P	0.000	0.000	0.000	0.000	0.000

Figures



a



b

Figure 1

The relation between vascular endothelial ferroptosis and dose of Ang II. All P values were calculated using Student's t-test and one-way analysis of variance (ANOVA). Detailed statistical tests are described in the Methods. Raw data are provided in Table 1, 2. a. Western blot analysis of ALOX12 VEGFA P53 in HUVECs to be treated with different dose of Ang II for 48hours. The experiments were repeated twice, independently, with similar results. Error bars are mean \pm s.d., n=3 independent experiments. b. The level of GSH and MDA was measured by GSH and MDA quantification kit in in HUVECs with different dose of Ang II treatment. Error bars are mean \pm s.d., n=3 independent experiments.

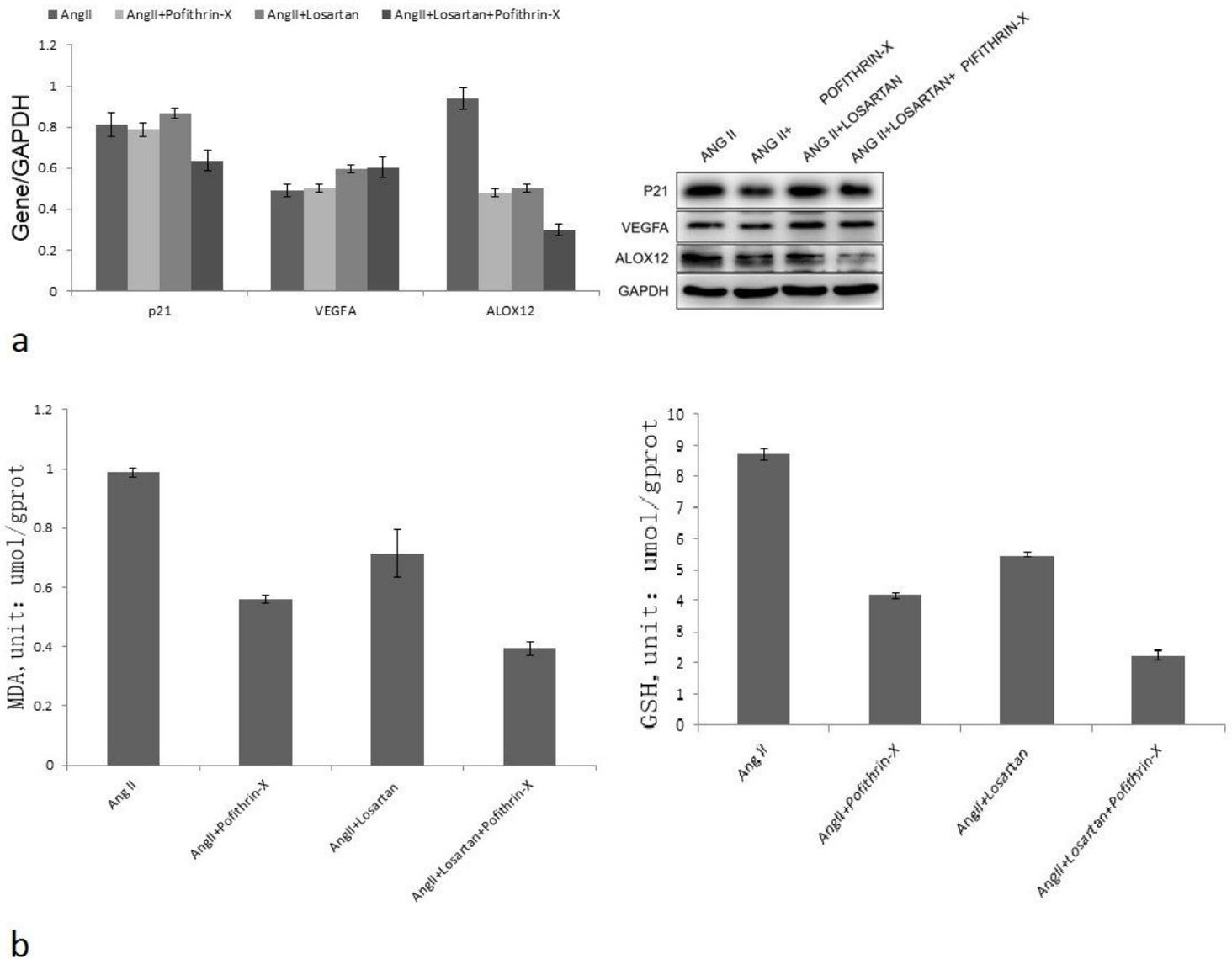


Figure 2

Losartan or/and Pofithrin-X prevented Ang-II- induced reduction of MDA, GSH, P21, ALOX12 and an increase in expression of VEGFA. All P values were calculated using Student’s t-test and one-way analysis of variance (ANOVA). Detailed statistical tests are described in the Methods. Raw data are provided in Table 3. a. Western blot analysis of HUVECs pre-incubated with AngII then treated with Losartan or/and Pofithrin-X as indicated for 48h. The experiments were repeated twice, independently, with similar results. Error bars are mean±s.d., n=3 independent experiments. b. Effect of Losartan or/and Pofithrin-X on MAD and GSH in HUVECs. The level of GSH and MDA was measured by GSH and MDA quantification kit in HUVECs pre-incubated with AngII then treated with Losartan or/and Pofithrin-X as indicated for 48h. Error bars are mean±s.d., n=3 independent experiments.