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Phytoestrogen α-Zearalanol Antagonizes Oxidized LDL-Induced Inhibition of Nitric Oxide Production and Stimulation of Endothelin-1 Release in Human Umbilical Vein Endothelial Cells

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Oxidative modification of low-density lipoprotein (LDL) leads to formation of the atherogenic molecule oxidized LDL (oxLDL), which is considered to be an important mediator for vascular endothelial dysfunction and atherosclerosis. It is speculated that reduced nitric oxide (NO) release/bioavailability and enhanced release of endothelin-1 (ET-1) may contribute to oxLDL-induced endothelial dysfunction. Estrogen may improve lipid profile and inhibit oxLDL-induced endothelial damage. However, estrogen replacement therapy has been suspended due to uncertainty in benefits versus risk (such as cancer progression) in postmenopausal women. This study was designed to evaluate the effect of a novel phytoestrogen, α-zearalanol (α-ZAL), on oxLDL-induced effect on NO and ET-1 production in human umbilical vein endothelial cells (HUVEC). HUVEC were incubated with oxLDL (50 μg/mL) for 24 h in the absence or presence of α-ZAL (0–1000 n*M*), 17β-estradiol (E_2, 10 n*M*), or the E_2 receptor antagonist ICI182780 (1 μ*M*). Levels of NO and ET-1 were measured by spectrophotometry and enzymatic immunoassay, respectively. NOS activity was evaluated by conversion of ^{3}H-arginine to ^{3}H-citrulline. Protein and mRNA expression of NOS and ET-1 were measured by Western blot and RT-PCR. Our results indicated that oxLDL significantly reduced NO release and NOS activity, and enhanced ET-1 production associated with reduced NOS3 (but not NOS2) expression and enhanced ET-1 mRNA expression. All these oxLDL-induced alterations were significantly attenuated or abolished by co-incubation with α-ZAL or E_2, both through an E_2 receptor-dependent mechanism. α-ZAL, E_2, and ICI182780 had no effect on NO/ET-1 release, NOS activity, or expression of NOS and ET-1. These data suggested that the phytoestrogen α-ZAL, like E_2, may effectively antagonize oxLDL-induced decrease in NO and increase in ET-1, which may be protective for endothelial function.

Key Words: α-Zearalanol; estrogen; oxLDL; nitric oxide; endothelin-1.

Introduction

Both lipid and apoprotein moieties of low-density lipoprotein (LDL) may be the likely targets for reactive oxygen species (ROS) and subsequently undergo oxidative modification to form oxidized LDL (oxLDL). A plethora of studies have depicted that oxLDL may promote apoptosis involving both mitochondrial and death receptor pathways such as Fas/FasL and TNF receptors *(1)*. As a result, endothelial function often becomes compromised with accumulation of oxLDL, which is known to lead to vasoconstriction, thrombosis, inflammation, atherosclerosis, myocardial infarction, and stroke *(1–3)*. Being a highly injurious LDL oxidative product, oxLDL induces cellular dysfunction in a time- and concentration-dependent manner including inhibition of constitutive nitric oxide (NO) synthase (NOS) expression, induction of expression of adhesion molecule and lectin-like oxLDL receptor-induced matrix metalloproteinase *(4–6)*. The present strategy against oxLDL-associated endothelial dysfunction has been largely focused on how to antagonize the cytotoxicity of oxLDL and minimize the oxidation of LDL *(3,5)*.

Estrogen has been shown to provide protection against endothelial dysfunction with up-regulation of NO production and down-regulation of endothelin-1 (ET-1) generation, especially under pathological conditions such as atherosclerosis and hypertension *(7,8)*. Interaction between estrogen and vasoactive factors such as NO has been implicated to play an important role of the gender-related differences in the vasomotor activity of oxLDL and the lesser cardiovascular incidence (e.g., atherosclerosis) in premenopausal females than in males *(9)*. NO and ET-1 are a pair

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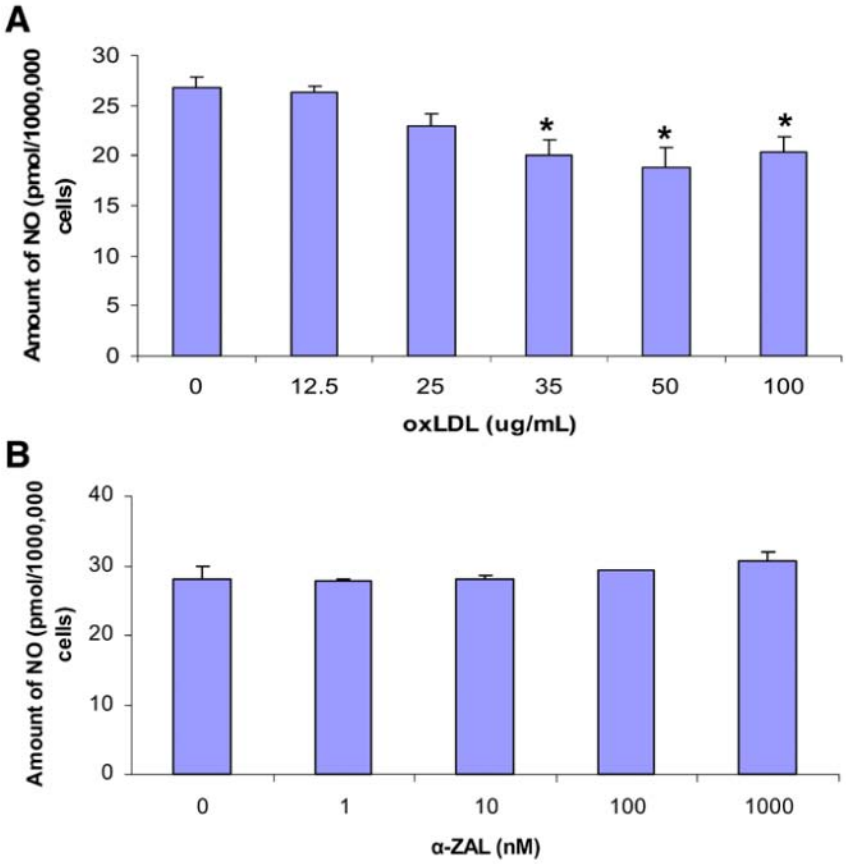

Fig. 1. Effect of oxLDL (**A**) and α-ZAL (**B**) on NO production. HUVEC were incubated with oxLDL (0–100 μg/mL) or α-ZAL (0–1000 n*M*) for 24 h. Levels of NO were determined using spectrophotometry. Mean ± SEM, $n = 4–6$ per data point, $*p < 0.05$ vs control (0 concentration of drug).

of endothelium-derived molecules with offsetting vasoactive properties. Their levels and functions may be disparately regulated by oxLDL, resulting in decreased NO and enhanced ET-1 levels, which lead to eventual endothelial damage and atherosclerosis development *(1,10,11)*.

Although estrogen has been shown to improve the lipid profile and inhibit oxLDL-induced stimulation of ROS generation and apoptosis in human umbilical vein endothelial cells (HUVEC) *(12,13)*, the beneficial effect of estrogen on endothelial function may be attributed to its capacity to keep balance of the NO/ET-1 pair "in check" against oxLDL *(12)*. Despite these indisputable beneficial effects for estrogen on endothelial function, estrogen replacement therapy is still quite controversial largely due to uncertainty in cardiovascular benefit and risk of developing breast as well as endometrial cancers in women taking estrogen replacement therapy *(14,15)*. Efficacious strategies to maximize estrogen-associated beneficial cardiovascular effects while minimizing estrogen-induced carcinogenesis are in high demand. Recently, plant-derived phytoestrogens have shown some promise as a replacement for estrogen to limit the risk of carcinoma while promoting the endothelial benefits of estrogen. The phytoestrogen genistein was shown recently to improve endothelial function in ovariectomized rats and postmenopausal women through increasing NO release while inhibiting that of ET-1 *(16,17)*. This study was designed to evaluate the effects of a novel phytoestrogen α-zearalanol (α-ZAL), a reductive product of the *Gibberella zeae* metabolite zearalenone, on basal- and oxLDL-induced endothelial release of NO and ET-1. Zearalenone is a member of the β-resorcylates and facilitates animal growth *(18)*. Recent evidence from our group indicated that α-ZAL efficiently reduces atherosclerosis development in the ovariectomized, cholesterol-fed rabbit model in a manner similar to estrogen without the estrogenous growth promoting property *(19)*.

Results

Effect of α-ZAL on Basal- and oxLDL-Induced Inhibition of NO Production and NOS Activity

Inhibition of NO synthesis and/or facilitation of NO inactivation have been shown as a major mechanism responsible for oxLDL-induced endothelial dysfunction *(5)*. To examine the possible impact of α-ZAL on oxLDL-induced endothelial function, we examined the effect of α-ZAL on basal- and oxLDL-induced inhibition of NO production or NOS activity in HUVEC. Results in Fig. 1 demonstrated that exposure of oxLDL (0–100 μg/mL) for 24 h to cultured HUVEC significantly depressed NO release at concentra-

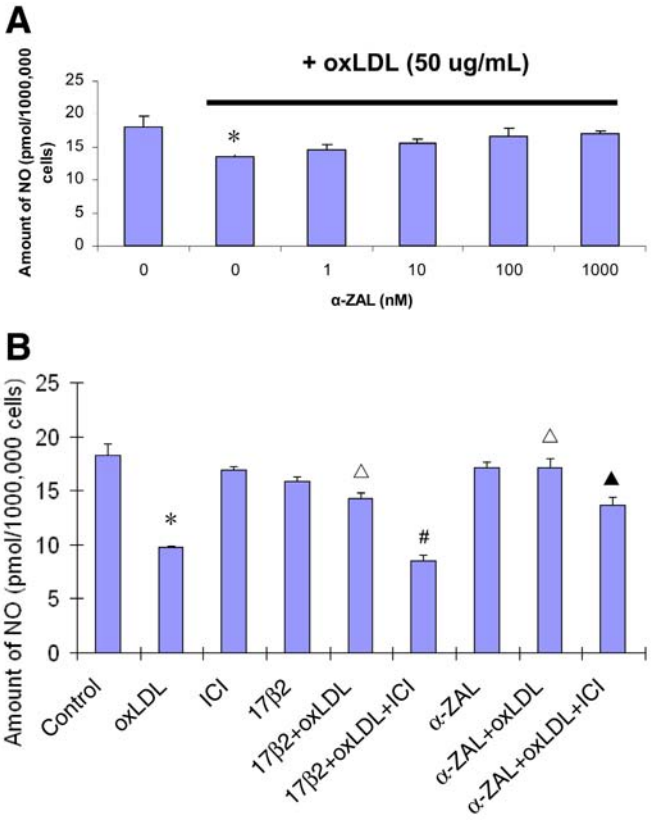

Fig. 2. Effect of α-ZAL and 17β E_2 on oxLDL-induced effect on NO production in HUVEC. (**A**) HUVEC were incubated with α-ZAL (0–1000 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL); (**B**) HUVEC were incubated with 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Mean ± SEM, n = 5–6 per data point, $^{*}p < 0.05$ vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL, $^{\blacktriangle}p < 0.05$ vs α-ZAL+oxLDL or oxLDL alone.

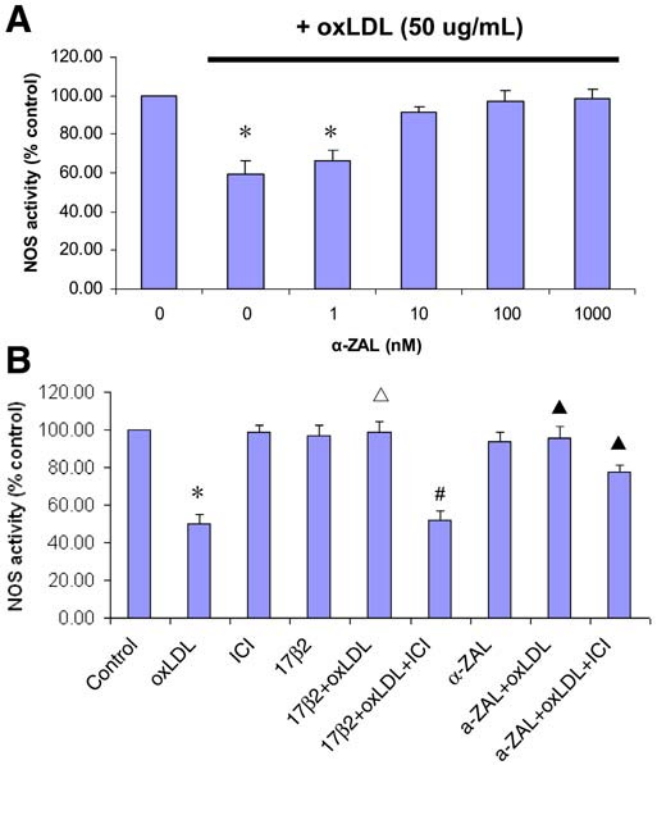

Fig. 3. Effect of α-ZAL and 17β E_2 on oxLDL-induced effect on NOS activity in HUVEC. (**A**) HUVEC were incubated with α-ZAL (0–1000 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL); (**B**) HUVEC were incubated with 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Mean ± SEM, n = 4 assays, $^{*}p < 0.05$ vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL, $^{\blacktriangle}p < 0.05$ vs α-ZAL+oxLDL or oxLDL alone.

tion 35–100 μg/mL, with an IC_{50} of 44.08 ± 17.82 μg/mL. This is consistent with an inhibition of NOS activity upon exposure to 50 μg/mL of oxLDL (Fig. 3). α-ZAL exhibited no overt effect on basal NO release (Fig. 1B) or NOS activity (Fig. 3) within the concentration range tested. However, it significantly attenuated oxLDL (50 μg/mL)-induced reduction in NO production or NOS activity, similar to that of E_2 (10 n*M*). The EC_{50}s for α-ZAL-elicit response against oxLDL-induced inhibition of NO production and NOS activity were 9.31 ± 1.78 n*M* and 6.41 ± 1.01 n*M*, respectively. E_2 did not affect basal NO release nor basal NOS activity. It should be noted that the protective action of α-ZAL against oxLDL (50 μg/mL)-induced inhibition of NO release or NOS activity is either absent or tends to be absent at the lowest concentration tested (1 n*M*). Both α-ZAL and E_2 elicited beneficial effects against oxLDL-induced decline in NO production or NOS activity may be cancelled by the E_2 receptor antagonist ICI182780 (1 μ*M*), depicting the involvement of E_2 receptor in the beneficial effect of α-ZAL and E_2 (Figs. 2 and 3). Interestingly, ICI182780 almost completely reversed the effect of E_2 on oxLDL-induced decrease of NO production/NOS activity but only partially attenuated the effect of α-ZAL on oxLDL-induced reduction in NO release or NOS activity (the "α-ZAL+oxLDL+ICI" groups were still significantly different from oxLDL groups in Figs. 2B and 3B), indicating that E_2 receptor is unlikely the sole receptor mediating the biological effect of α-ZAL. ICI182780 alone did not have any effect on NO release or NOS activity. Collectively, these data indicated the beneficial effect of α-ZAL against oxLDL-induced decrease in NO through, at least in part, an E_2 receptor-mediated mechanism, similar to that of E_2.

Effect of α-ZAL on Basal- and oxLDL-Induced ET-1 Production

In addition to a drop in NO level, ET-1 was reported to accumulate on oxLDL exposure, which may contribute to oxLDLinduced endothelial dysfunction *(20)*. We thus evaluated the effect of α-ZAL on basal- and oxLDL-induced ET-1 release in HUVEC. Results in Fig. 4 demonstrated

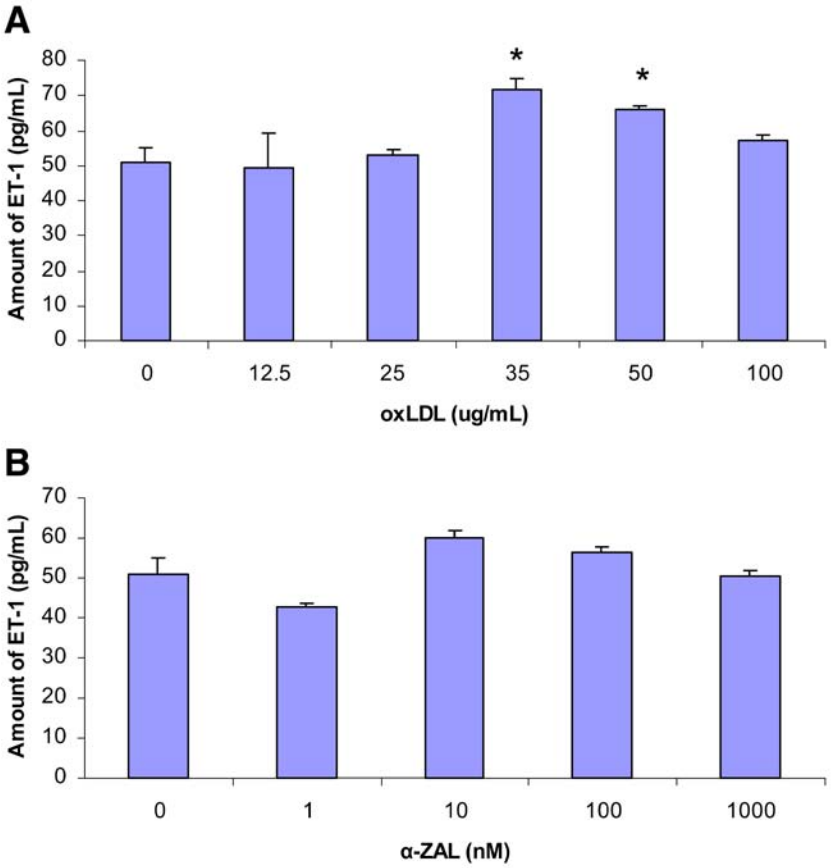

Fig. 4. Effect of oxLDL (**A**) and α-ZAL (**B**) on ET-1 production. HUVEC were incubated with oxLDL (0–100 μg/mL) or α-ZAL (0–1000 n*M*) for 24 h. Levels of ET-1 were determined using the Enzyme Immunometric Assay. Mean ± SEM, $n = 5–6$ per data point, $^*p < 0.05$ vs control (0 concentration of drug).

that exposure of oxLDL (0–100 μg/mL) for 24 h to HUVEC significantly enhanced ET-1 release at concentration 35–100 μg/mL, with an EC_{50} of 37.75 ± 26.56 μg/mL. However, the highest concentration of oxLDL (100 μg/mL) failed to elicit any significant effect on ET-1 release. Although the precise mechanism is not available for this negative finding at 100 μg/mL oxLDL, possible non-specific effects of high oxLDL may contribute to "cancel off" its direct action on ET-1 release. Although α-ZAL exhibited no effect on basal ET-1 release within the concentration range tested (1–1000 n*M*) (Fig. 4B), it (at concentrations of 1–1000 n*M*) did significantly attenuate oxLDL (50 μg/mL)-induced increase in ET-1 production, similar to that of E_2 (10 n*M*). The IC_{50} of α-ZAL-induced inhibition of oxLDL-triggered ET-1 release was 7.89 ± 19.42 n*M*. Lower concentration of α-ZAL (0.1 n*M*) failed to elicit such attenuation against oxLDL-induced enhancement of ET-1 release (data not shown). E_2 did not affect the basal ET-1 release. Similar to their effects on NO/NOS system, both α-ZAL and E_2-elicited effects against oxLDL-induced increase in ET-1 release were antagonized by the E_2 receptor antagonist ICI182780 (1 μ*M*), supporting the notion of involvement of E_2 receptor α-ZAL and E_2-elicited beneficial effect (Fig. 5). It is worthy mentioning that ICI182780 completely reversed the effect of E_2 on oxLDL-induced increase in ET-1 but failed to affect α-ZAL-induced effect on oxLDL-induced rise in ET-1 level. These data indicate that E_2 receptor is unlikely involved in the effect of α-ZAL against oxLDL-induced ET-1 release. ICI182780 itself did not have any effect on ET-1 release. These results suggested that α-ZAL may offset oxLDL-induced rise in ET-1, at least in part, through an E_2 receptor-independent mechanism.

Effect of α-ZAL on Basal- and oxLDL-Induced Response on NOS2 and NOS3

To identify the mechanism of action responsible for α-ZAL-induced antagonism against oxLDL-induced decrease in NO production, expression of NOS2 (mRNA) and NOS3 (both protein and mRNA) was measured in oxLDL (50 μg/mL)-treated cells in the absence or presence of α-ZAL (10 n*M*), E_2 (10 n*M*), or the E_2 receptor antagonist ICI182780 (1 μ*M*). Figures 6 and 7 show that oxLDL significantly depressed both protein and mRNA expression of endothelial NOS (NOS3). α-ZAL and E_2 themselves did not affect expression of NOS3 (protein or mRNA). Consistent with their action on NO release, both α-ZAL and E_2 reversed oxLDL-induced reduction of NOS3 expression, which was nullified by ICI182780. These data again favored an E_2 recep-

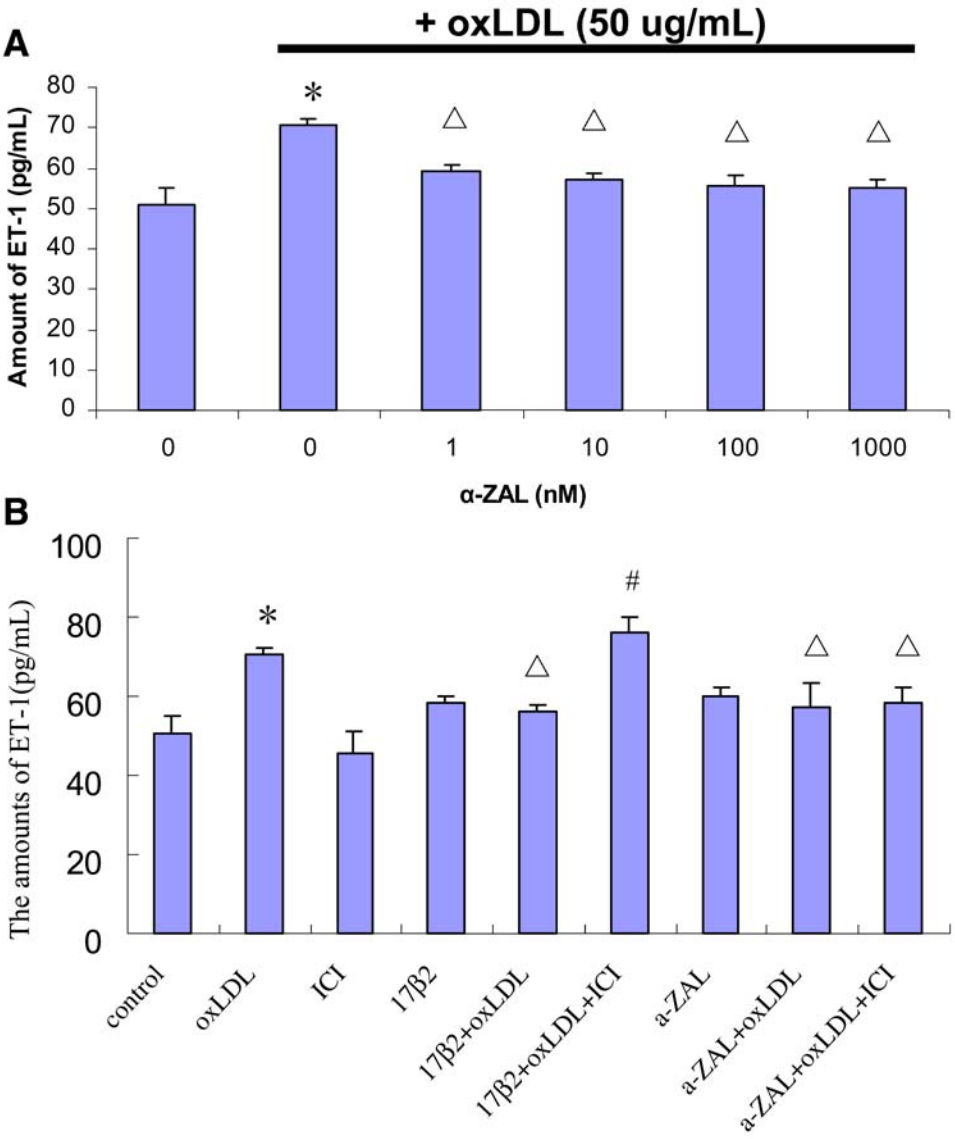

Fig. 5. Effect of α-ZAL and 17β E_2 on oxLDL-induced effect on ET-1 secretion in HUVEC. (**A**) HUVEC were incubated with α-ZAL (0–1000 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL); (**B**) HUVEC were incubated with 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Mean ± SEM, $n = 5$, $^{*}p < 0.05$ vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL.

tor-mediated mechanism in the protective response of α-ZAL and E_2 against oxLDL-induced inhibition of NO production. The fact that ICI182780 did not fully reverse the action of α-ZAL (Figs. 6 and 7) suggests that E_2 receptor is unlikely the sole receptor responsible for α-ZAL effect. ICI182780 itself did not have any effect on NOS expression. Neither oxLDL nor hormones (α-ZAL or E_2) affected the mRNA transcript of inducible NOS (NOS2) (Fig. 8), indicating unlikely involvement of NOS2 in oxLDL or hormone-induced response on NO release.

Effect of α-ZAL on Basal- and oxLDL-Induced Response of ET-1 mRNA Expression

Our results further revealed that oxLDL significantly enhanced mRNA expression of ET-1. While α-ZAL and E_2 themselves did not affect ET-1 expression, they both reversed oxLDL-induced increase in ET-1 expression, consistent with their effect on ET-1 release. ICI182780 itself did not affect the expression of ET-1. However, the E_2 receptor antagonist abolished the effect of E_2 but only partially (but significantly) attenuated that of α-ZAL on oxLDL-induced rise of ET-1 mRNA expression, suggesting that α-ZAL-elicited response on oxLDL-induced ET-1 expression is partially mediated through the E_2 receptor (Fig. 9).

Discussion

Our results demonstrated that the phytoestrogen α-ZAL prevented oxLDL-induced inhibition of NO production, depression of NOS activity, and elevation of ET-1 release

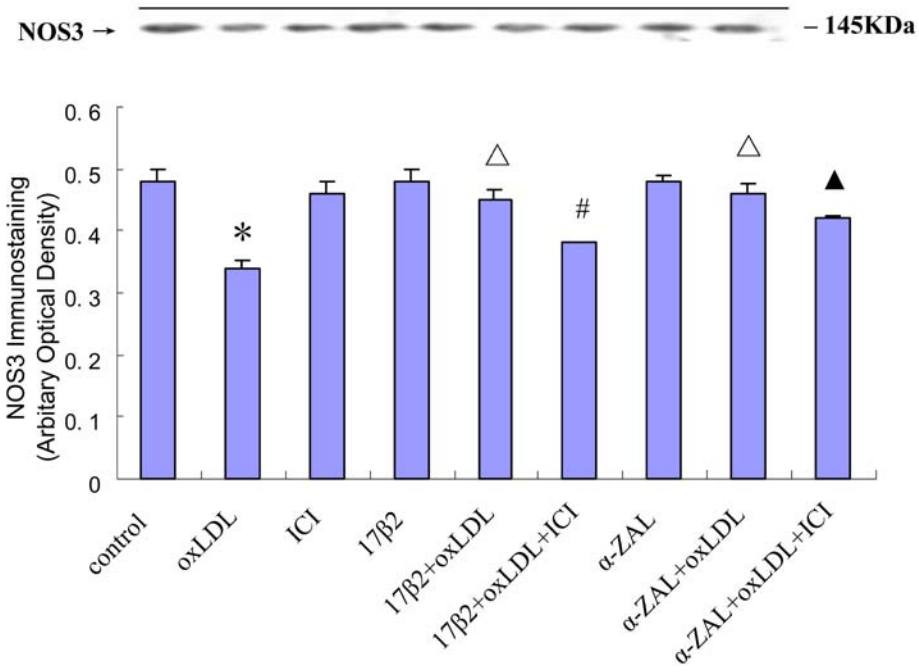

Fig. 6. Effect of 17β E_2 and α-ZAL on NOS3 protein expression. HUVEC were incubated with either 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. NOS3 protein expression was evaluated with Western blotting. Insets show representative gel blots following the same sequence of group order as bar graph. Mean ± SEM, $n = 3$, $^{*}p < 0.05$ vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL, $^{\blacktriangle}p < 0.05$ vs α-ZAL+oxLDL or oxLDL alone.

in HUVEC, in a manner similar to estrogen involving estrogen receptor(s). The α-ZALelicited beneficial effects against oxLDL-induced changes in NO and ET-1 appeared to be related to its ability to prevent oxLDL-induced down- and up-regulation of *NOS*3 and *ET*-1 gene, respectively, also similar to estrogen. These observations provided compelling support to earlier reports from our lab as well as others on possible protective mechanisms of α-ZAL against estrogen deficiency-associated endothelial dysfunction and cholesterol diet-induced atherosclerosis involving estrogen receptor and NO *(19,21)*. Our current results, in conjunction with those published earlier *(19,21)*, suggest that α-ZAL may possess favorite endothelial properties and be used as a replacement for estrogen in the treatment of endothelial dysfunction in postmenopausal women. This is in line with the finding that α-ZAL prevents postmenopausal bone loss and reverses ovariectomy-induced endothelial dysfunction similar to estrogen *(21,22)*. Data from our present study not only provided evidence that α-ZAL directly antagonized oxLDL-induced decrease of NO release or NOS activity, but also implicated the involvement of another key endothelial regulator, ET-1. Elevated ET-1 level has been directly associated with endothelial dysfunction which can be suppressed by estrogen replacement therapy *(23,24)*. The observation that α-ZAL itself did not affect production of NO, NOS activity, and ET-1 at basal level indicated the phytohormone is unlikely to interfere with normal endothelial function.

Atherosclerosis is a major risk factor for myocardial infarction and stroke. One of the main contributing factors for atherosclerosis is the loss of balance of among vasoactive factors, out of which NO and ET-1 probably have drawn the most attention *(25)*. The levels of NO and ET-1 have been demonstrated to closely correlate with the atherosclerotic process and other vasculopathies *(25,26)*. NO is a potent vasodilator exerting its most important endothelial protection against the endothelial injury and development of atherosclerosis *(26)*. On the other hand, the role of ET-1 in atherosclerosis may extend over a wide variety of physiological actions including vasoconstriction, mitogenesis, neutrophil adhesion, platelet aggregation, and hypertrophy, as well as its propensity to induce ROS formation *(25)*. The imbalance between NO and ET-1 is believed to contribute to oxLDL-induced endothelial dysfunction, thrombosis, and atherosclerotic injury *(4–6)*. Our data suggested that oxLDL-induced loss of NO production and reduction of NOS activity in HUVEC may be related to reduced NOS3 rather than the inducible NOS2 mRNA expression, consistent with previous report *(27)*. On the other hand, it was reported that oxLDL may promote the expression of mRNA and protein of ET-1 and stimulate ET-1 release in endothelial cells *(28)*, consistent with our current finding. Our data suggested that the mechanism of action behind α-ZAL-induced protective effect against oxLDL-elicited changes in NO and ET-1 release from endothelial cells is likely to be attributed by the up- and down-regulatory effect of α-ZAL

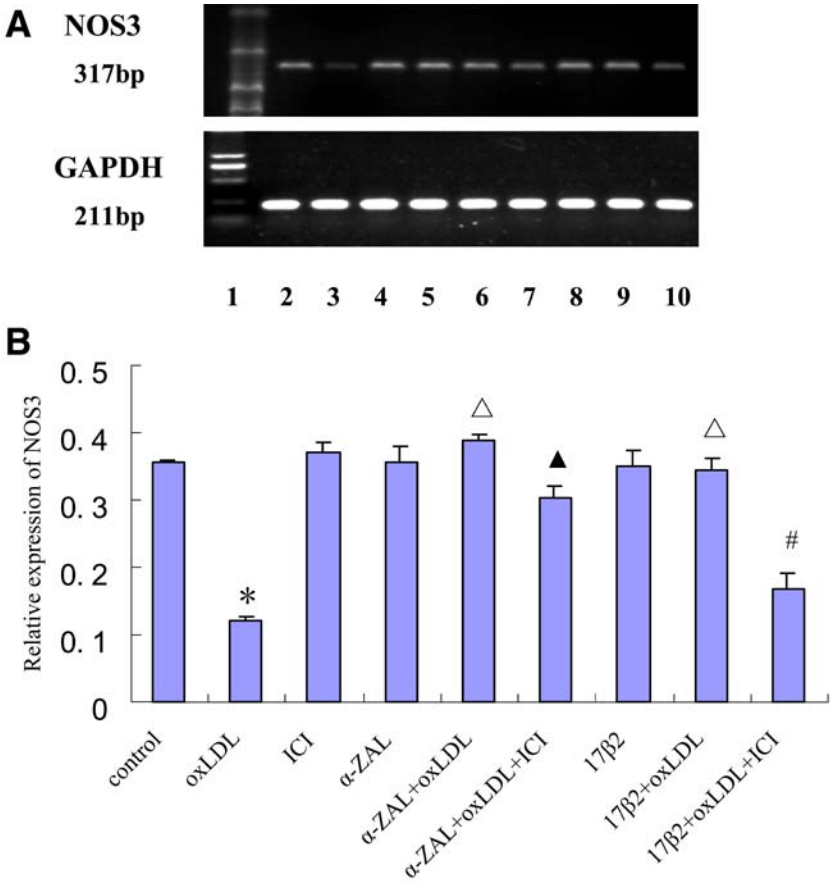

Fig. 7. Effect of 17β E_2 and α-ZAL on NOS3 mRNA expression in HUVECs. HUVEC were incubated with either 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Total RNA was extracted and RT-PCR was performed for analysis of NOS3 mRNA expression (normalized to that of GAPDH). The PCR products were analyzed by 2% agarose gel electrophoresis. The predicted size of RT-PCR products for NOS3 and GAPDH are 317 bp and 211 bp, respectively. Lane 1=marker(DL2000); Lane 2=control; Lane 3=oxLDL; Lane 4=ICI182780; Lane 5=α-ZAL; Lane 6= α-ZAL+oxLDL; Lane 7=α-ZAL+oxLDL+ICI; Lane 8=E_2; Lane 9=E_2+oxLDL; Lane 10=E_2+oxLDL+ICI. Mean ± SEM, $n = 3$, *p<0.05 vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL, $^{\blacktriangle}p < 0.05$ vs α-ZAL+oxLDL or oxLDL alone.

on oxLDL- (not basal) induced expression of NOS3 and ET-1 genes, in a manner similar to estrogen.

Data from our present in vitro study on the protective action of α-ZAL against oxLDL-induced imbalance of NO/ET-1 pairs may add support to our recent in vivo finding using ovariectomized, cholesterol-fed atherosclerotic rabbits *(19)*. Following 12 wk of high cholesterol feeding with or without α-ZAL or estrogen supplementation, the total vascular area of the high cholesterol diet–induced atherosclerotic plaque formation may be reduced by approx 75% with dietary supplementation of α-ZAL, which was as equally effective as estrogen. The plasma lipid total cholesterol, triglycerides, low-density lipoprotein-cholesterol, and Apo-protein B levels were also reduced by various degrees following α-ZAL treatment compared to the high cholesterol diet group *(19)*. The protective action of α-ZAL against oxLDL-induced imbalance of NO and ET-1 suggests that α-ZAL may offer beneficial action on endothelial and/or vascular function through mechanisms other than lipid lowering effect. Endothelial dysfunction resulted from oxLDL accumulation is a key hallmark in the pathogenesis of atherosclerosis *(1–3)*. Keeping the NO and ET-1 pairs in balance is essential for normal endothelial and/or vascular function, which may be either dependent or independent of the overall lipid profile or lipid oxidation (e.g., oxLDL levels) *(2–5,28)*.

One rather interesting finding from our current study was that α-ZAL-induced response on NO production, NOS activity, and NOS3 expression was only partially mediated through the estrogen receptor, whereas its response on ET-1 release and ET-1 expression seemed to be independent of the estrogen receptor. This is different from the response from estrogen, which depends solely on estrogen receptor This apparent discrepant response of α-ZAL on NO production and ET-1 release seems to indicate the phytoestrogen may use another receptor independent of estrogen receptor (not sensitive to ICI182780) for its inhibitor action on NO or ET-1 system. However, it may also be argued that the disparity between α-ZAL and estrogen in the ICI182780 response

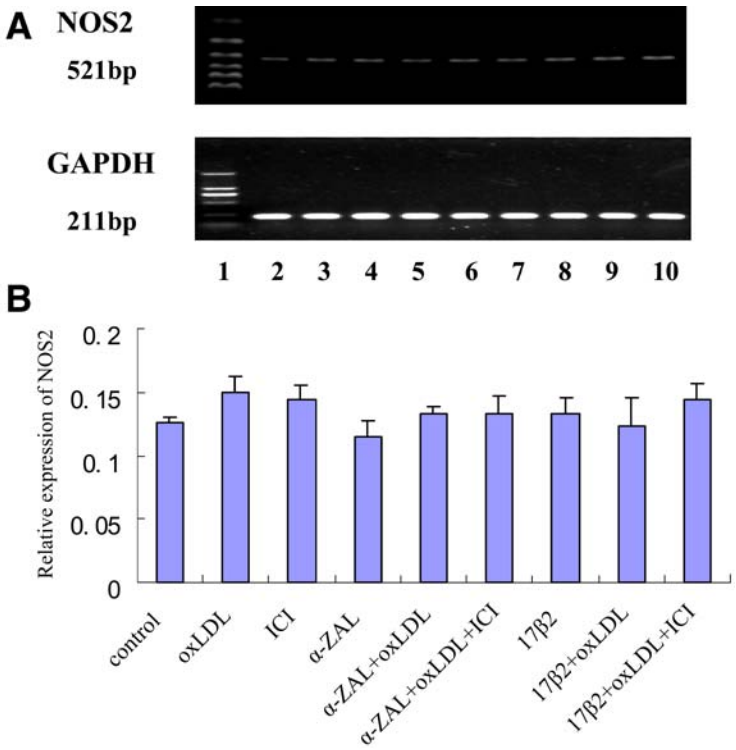

Fig. 8. Effect of 17β E_2 and α-ZAL on NOS_2 mRNA expression in HUVECs. HUVEC were incubated with either 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Total RNA was extracted and RT-PCR was performed for analysis of NOS2 mRNA expression (normalized to that of GAPDH). The PCR products were analyzed by 2% agarose gel electrophoresis. The predicted size of RT-PCR products for NOS2 and GAPDH are 521 bp and 211 bp, respectively. Lane 1=marker(DL2000); Lane 2=control; Lane 3=oxLDL; Lane 4=ICI182780; Lane 5=α-ZAL; Lane 6= α-ZAL+oxLDL; Lane 7=α-ZAL+oxLDL+ICI; Lane 8=E_2; Lane 9=E_2+oxLDL; Lane 10=E_2+oxLDL+ICI. Mean ± SEM, n = 3.

may be due to the difference in binding affinity to estrogen receptor. Although there seems to be similarity in the binding of α-ZAL and estrogen to hepatic estrogen receptor in vitro *(29)*, the affinity of binding to the estrogen receptor for α-ZAL in the uterus and cardiovascular systems is significantly less than that of estrogen (Dai et al., unpublished data). This is supported by our recent finding of discrepant cardiac contractile response in ventricular myocytes between α-ZAL and estrogen *(30)*. Further study is warranted to examine the specific receptor types involved in the endothelial response of α-ZAL. In addition, oxidative stress is commonly seen under conditions of enhanced atherosclerosis and LDL oxidation *(1–3)*. Our preliminary data indicated that α-ZAL may effectively protect against the oxidative stress inducer homocysteine-induced decrease in NO, increase in ET-1/NO ratio, and apoptosis in HUVEC (Duan, Dai, and Ren, unpublished data), suggesting a causal relationship between α-ZAL-elicited protection against homocysteine- and oxLDL-elicited cellular injury.

Zearalenone, the parent compound of α-ZAL, is present in plants and vegetables such as wheat, cotton, corn, celery, carrots, and beets. As an endogenous hormone, zearalenone and its reductive metabolite α-ZAL are believed to be much safer than estrogen and play a significant role in herbal development and growth *(18)*. During the past two to three decades, hormones from natural resources have drawn significant attention essentially due to their properties to reduce the overall cardiovascular morbidity and mortality *(31,32)*. In addition, our preliminary evidence suggested that α-ZAL enlarged the ovariectomized uterus and mammary gland to a much lesser extent than estrogen *(33)*. No obvious adverse effects or organ pathological alterations were observed in animals fed with dietary α-ZAL *(19,33)*, suggesting the safety issue of α-ZAL may not be a major concern.

Although estrogen may benefit cardiovascular function, blood lipid profile, vascular resistance, blood pressure, and oxidative stress *(34)*, recent clinical trials such as the Heart and Estrogen/Progestin Replacement Study (HERS) revealed unpredicted or even surprising findings regarding the cardiovascular action of estrogen, independent of its apparent adverse effect on growth and tumorogenesis *(35)*. Rather than offering any cardiovascular protection, it has been suggested that estrogen may in fact deteriorate certain cardiovascular function in women with established heart diseases *(35,36)*. With the pros and cons of estrogen and estrogen replacement therapy on cardiovascular health unclear and debatable, the National Institutes of Health has put on hold large clinical trials of estrogen replacement therapy and the

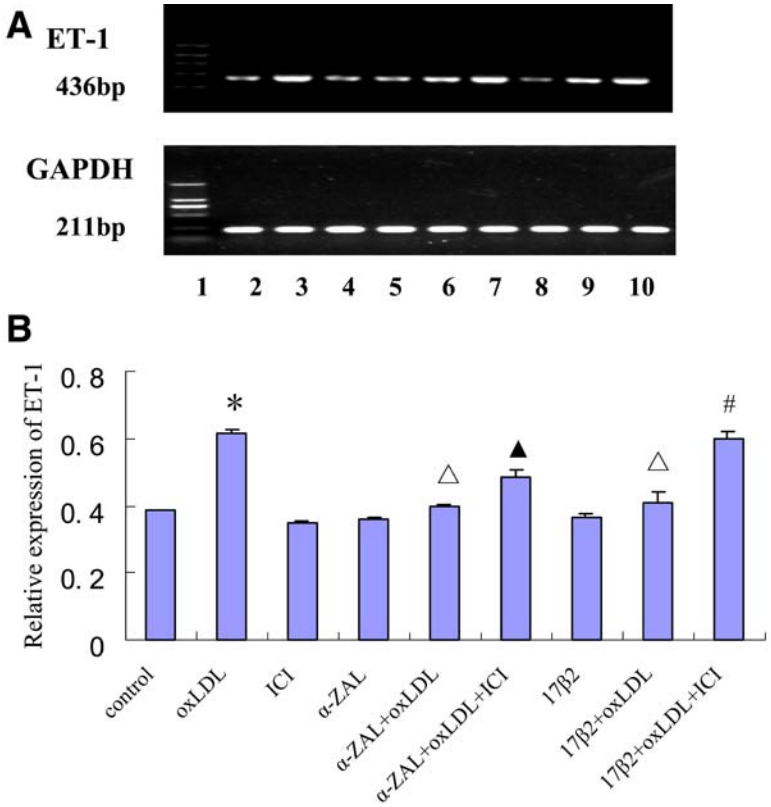

Fig. 9. Effect of 17β E_2 and α-ZAL on ET-1 mRNA expression in HUVECs. HUVEC were incubated with either 17β E_2 (10 n*M*) or α-ZAL (10 n*M*) for 24 h in the absence or presence of oxLDL (50 μg/mL) or the E_2 receptor antagonist ICI182780 (1 μ*M*), or both. Total RNA was extracted and RT-PCR was performed for analysis of ET-1 mRNA expression (normalized to that of GAPDH). The PCR products were analyzed by 2% agarose gel electrophoresis. The predicted size of RT-PCR products for NOS3 and GAPDH are 436 bp and 211 bp, respectively. Lane 1=marker(DL2000); Lane 2=control; Lane 3=oxLDL; Lane 4=ICI182780; Lane 5=α-ZAL; Lane 6= α-ZAL+oxLDL; Lane 7=α-ZAL+oxLDL+ICI; Lane 8= E_2; Lane 9=E_2+oxLDL; Lane 10=E_2+oxLDL+ICI. Mean ± SEM, $n = 3$, $^{*}p < 0.05$ vs control (0 concentration drug), $^{\Delta}p < 0.05$ vs oxLDL group, $^{\#}p < 0.05$ vs 17β E_2+oxLDL, $^{\blacktriangle}p < 0.05$ vs α-ZAL+oxLDL or oxLDL alone.

American Heart Association warned women of the risk of coronary heart disease with estrogen replacement therapy *(36)*. The general consensus is that women should not consider estrogen replacement therapy without any known benefits of estrogen on cardiovascular function *(35,36)*. Thus, the potential clinical value of phytoestrogens has to be evaluated not only from their growth promoting effects but also from the perspective of their cardiovascular safety. Our preliminary evidence suggested that the uterine enlargement elicited by α-ZAL was only approx 20% of that associated with equivalent doses of estrogen. The uterus and mammary gland displayed little pathological change compared to treatment with estrogen *(33)*. However, the potential cardiovascular risk and adverse effects have not been elucidated for α-ZAL and is in need of further investigation. Over a hundred kinds of phytoestrogens have been identified since the 1950s, with genistein and isoflavone being most common *(31,37)*. However, certain phytoestrogens have shown adverse effects on cardiovascular function, which jeopardizes their clinical potential and usage *(37)*.

In summary, our data exhibited protection of α-ZAL against oxLDL-induced decrease in NO release, NOS activity, and increase in ET-1 production in endothelial cells through up-regulation of NOS3 (not NOS2) and down-regulation of ET-1 genes under oxLDL exposure, in a manner similar to that of estrogen. These beneficial effects of α-ZAL seem to be mediated, at least in part, through the estrogen receptor although other receptor involvement cannot be excluded at this time. These findings indicate the therapeutic application of this phytoestrogen in the treatment and prevention of endothelial diseases. Future research on zearalenone and its derivative α-ZAL may be focused on receptor binding property, potential adverse effects such as tumor genesis and in cardiovascular system, its pharmacological comparison with estrogen, and the molecular mechanism of its action.

Materials and Methods

HUVEC Culture

Confluent HUVEC (HUVEC, ATCC, Manassas, VA) with a density of approx 10^5 cells/mL were maintained in Medium 199 containing 10 m*M* HEPES, 1 m*M* glutamine, 0.1 mg/mL heparin, 100 μg/mL crude endothelial cell growth supple-

ment, 100 unit/mL penicillin, and 100 μg/mL streptomycin (pH 7.4) supplement and 10% fetal bovine serum at 37°C with 5% CO_2. The second through the fifth passage of HUVEC was used at subconfluence. The HUVEC were preincubated with oxLDL at designated concentrations with or without α-ZAL (0–1000 n*M*), 17β-estradiol (E_2, 10 n*M*), or the E_2 receptor antagonist ICI182780 (1 μ*M*) for 24 h in fresh medium. The concentration range chosen for α-ZAL was largely based on previous studies using in vivo rabbit model of atherosclerosis *(19)* and in vitro cardiomyocytes *(30)*. The supernatant of HUVEC culture medium was collected to determine the levels of NO and ET-1. At 16–24 h before hormonal treatment, cells were incubated with phenol red-free M199 without serum (wM199).

Preparation of oxLDL

Human LDL was isolated through sequential ultracentrifugation. oxLDL was prepared by dialysis of 500 μg/mL of LDL in PBS containing 5 μ*M* $CuSO_4$ for 12 h at 37°C. LDL oxidation was terminated by excessive dialysis to remove Cu^{2+}, refrigeration at 4°C, and addition of 0.3 m*M* EDTA. The product was then sterilized by filtration through a 0.22-μm filter and stored under nitrogen gas at 4°C. Protein content was determined by the Bradford assay. The degree of oxidation was assessed with increased mobility on agarose gel (2.5 to 3.0 vs native LDL) and thiobarbituric acid-reactive substances (TBARS). LDL had TBARS value of less than 1 nmol/mg, whereas oxLDL had TBARS value between 10 and 30 nmol/mg. All lipoproteins were used within 3 wk after initial preparation *(38,39)*.

Measurement of NO

NO was spectrophotometrically assayed by measuring the accumulation of its stable degradation products, nitrate and nitrite using a colorimetric NO assay kit (Oxford Biomedical Research, Oxford, MI) *(40)*. Briefly, 5 μL of supernatant of cultured HUVEC was added into 96-well plate. Buffer containing 50 m*M* MOPS/1 m*M* EDTA was added into each well to bring the volume to 85 μL; 5 μL of the reconstituted nitrate reductase and 10 μL of 2 m*M* NADH were then added into each well before the 96-well plate was shaken for 20 min at room temperature. Ten microliters of color reagent #1 (*p*-aminobenxenesulfonamide dissolved in 3 *M* HCl) was then added and shaken briefly before 50 μL color reagent #2 [*N*-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in deionized H_2O] was added and shaken for another 5 min at room temperature. Absorbance value was obtained at 540 nm in microfilter plate reader.

Measurement of ET-1

The level of ET-1 was determined in HUVECs using a human ET-1 enzyme immunometric assay kit (Assay Designs, Inc. Ann Arbor, MI) based on a double-antibody sandwich technique *(41)*. The assay was specific for ET-1, ET-2, and ET-3. However, only ET-1 is secreted from and may be measured in the culture supernatant of endothelial cells. The detection threshold for ET-1 was 0.14 pg/mL. The interassay and intraassay coefficients of variation were below 3.3%.

Western Blot

HUVEC were lysed in 20 m*M* Tris (pH 7.4) containing 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X, 0.1% SDS and 1% sodium deoxychlorate, 1 m*M* phenylmethylsulfonyl fluoride, 50 m*M* sodium fluoride, 1 m*M* sodium orthvanadate, 50 μg/mL aprotinin, and 50 μg/mL leupeptin. A total of 50 μg of extracted protein was loaded to 15% SDS-polyacrylamide gel. Proteins were transferred to PVDF sequi-blot membranes (BioRad, Hercules, CA). Nonspecific binding sites were blocked for 2 h with 5% nonfat dried milk in TBS (20 m*M* Tris, 0.5 *M* NaCl, pH 7.5). The membranes were incubated overnight at 4°C with anti-NOS3 antibody (1:1000 dilution, anti-rabbit, from Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membranes were incubated for 2 h at room temperature with secondary antibody. The antigens were detected by the luminescence method using Super-Signal West Dura Extended Duration Substrate (Pierce Co., Rockford, IL). After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad densitometer (Model GS-800) *(42)*.

NOS Activity

NOS activity was evaluated by the ^{3}H-arginine to ^{3}H-citrulline conversion assay as described *(42)*. Following 24 h of incubation with oxLDL (50 μg/mL) in the absence or presence of α-ZAL (10 n*M*) or E_2 (10 n*M*), HUVEC were placed in Hanks' balanced salt solution (HBSS) medium containing 1 μCi/mL ^{3}H-arginine with Trasylol (0.2 kIU/mL). The cells were incubated for 30 min before the reaction was terminated by aspiration of the incubation medium and replacement with iced HBSS containing 5 m*M* L-arginine and 4 m*M* EDTA. Cells were lysed with 20 m*M* Tris and centrifuged. An aliquot of the supernatant was diluted with 1:1 (v/v) H2O/Dowex-50W (20–50, 8% cross-linked), and loaded on a polypropylene BioRad EconoColumn. The effluent was collected and ^{3}H-citrulline was counted by scintillation.

RNA Isolation and RT-PCR Amplification

Total RNA from HUVECs was isolated with TRIzol (Invitrogen, Carlsbad, CA). First-strand cDNA templates were created in final reaction conditions containing 2 μg of total RNA, 500 ng of oligo(dT), 10 m*M* dNTPs, 100 m*M* dithiothreitol, and 50 units of superscript reverse transcriptase (Invitrogen). Primers were synthesized according to motif: GCAGCCTCACTCCTGTTTT (NOS3, sense), CACCACG TCATACTCATCCA (NOS3, antisense); AGCGGTAACA AAGGAGATAG (NOS2, sense), CCCGAAACCACTCGT ATT (NOS2, antisense); CGTTGTTCCGTATGGACTTG (ET-1, sense), AGGCTATGGCTTCAGACAGG (ET-1, antisense); ACGGATTTGGTCGTATTGGG (*GAPDH*, sense), TCCTGGAAGATGGTGATGGG (*GAPDH*, antisense).

After denaturing at 94°C for 5 min, PCR amplification was performed for 30 cycles (94°C for 30 s, 53°C for 30 s, and 72°C for 60 s), followed by a final extension step (72°C for 10 min). PCR products were analyzed by 2% agarose gel electrophoresis. The housekeeping gene *GAPDH* was used as a control template for normalizing relative changes of NOS3, NOS2, ET-1 mRNA in RT-PCR.

Data Analysis

For each experimental series, data are presented as mean ± SEM. Statistical significance ($p < 0.05$) for each variable was estimated by one-way analysis of variance (ANOVA), followed by Fisher's test.

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