Phytoestrogen α-zearalanol inhibits homocysteine-induced endothelin-1 expression and oxidative stress in human umbilical vein endothelial cells

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Abstract

Although estrogen replacement therapy may improve dampened endothelial function in postmenopausal women, the associated risk of breast and ovarian cancer has limited its long-term use. Identifying effective alternative remedy with less carcinogenicity is in serious demand. This study was designed to examine the effect of the phytoestrogen α-zearalanol (α-ZAL) on homocysteine-induced endothelin-1 (ET-1) induction, reactive oxygen species (ROS) production and transcription pathways in human umbilical vein endothelial cells (HUVECs). ROS was measured by DCF fluorescent microscopy. Homocysteine-induced expression of ET-1 mRNA, ERK, pERK and c-jun/AP-1 protein was measured using RT-PCR and Western blot analysis, respectively. ET-1 secretion was determined by the enzymatic immunoassay. Transcriptional factor AP-1 expression in response to α-ZAL, homocysteine or both was evaluated by transient transfection assay. Our data revealed that α-ZAL ablated homocysteine-elicited ET-1 secretion, upregulated ET-1 mRNA and homocysteine-induced ROS accumulation without any effects by itself. α-ZAL also nullified homocysteine-induced increase in c-Jun/AP-1 expression/activity without eliciting any effect by itself. Collectively, our data indicated that α-ZAL may antagonize homocysteine-induced ET-1 gene induction, ROS accumulation, activation of ERK signaling pathway and AP-1 transcriptional factor, all of which may contribute to α-ZAL-induced beneficial effect on endothelial function.

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1. Introduction

Hyperhomocysteinemia is a well-known independent risk factor for cardiovascular diseases such as ischemic heart diseases, stroke, peripheral vascular disease, atherosclerosis and postmenopausal syndrome [1]. Homocysteine elicits a cascade of vascular wall injuries including chemical modification of lipoproteins, alteration of vascular structure, oxidative stress, endothelial dysfunction and repair incompetence as well as proliferation of vascular smooth muscle cells [2]. It has been demonstrated that elevated homocysteine levels are closely associated with accumulation of reactive oxygen species (ROS), which is believed to play a key role in homocysteine-induced endothelial dysfunction, vascular damage and pathogenesis of atherosclerosis [3].

Estrogen replacement therapy has been clinically used to lower certain cardiovascular risks in postmenopausal women including homocysteine-induced endothelial dysfunction and vascular damage [4]. Nonetheless, risk of developing breast as well as endometrial cancers in women taking estrogen replacement therapy has largely limited its clinical application [5]. Efficacious strategies to promote estrogen-associated beneficial cardiovascular effects while
minimizing estrogen-induced carcinogenesis are in high demand. Recently, evidence from epidemiological, clinical as well as experimental studies has implicated that the plant-derived phytoestrogens display promises as a replacement for estrogen to prevent atherosclerosis, osteoporosis, carcinogenesis and Alzheimer’s disease [8]. Li and colleagues first isolated α-zearalanol (α-ZAL), a reductive product of the fungus Gibberella Zeae metabolite zearalanone, from culture medium of zearalanone and identified it as a member of the β-resorcylate family. Both α-ZAL and its parent compound zearalenone have been shown to act as universal endogenous hormones for plant growth with α-ZAL being more potent and less toxic. Our group has demonstrated that α-ZAL retards development of atherosclerosis with little undesired effects on the growth of mammary gland and uterine [6]. Nevertheless, the precise mechanism behind the beneficial effect of α-ZAL on vascular endothelium has not been fully elucidated. The aim of the present study was to examine the effects of α-ZAL on homocysteine-mediated regulation of endothelin-1 (ET-1) gene expression, generation of ROS and transcription pathways in human umbilical vein endothelial cells (HUVECs). ET-1, a 21-amino acid polypeptide, is by far the most potent vasoconstrictive peptide. ET-1 has been shown to play a key role in the pathogenesis of atherosclerosis [7]. Our earlier report revealed that α-ZAL may suppress homocysteine-elicted ET-1 secretion from vascular endothelial cells [9]. Elevated plasma homocysteine level is an independent risk factor for coronary, cerebral and peripheral vascular dysfunction manifested as impaired vascular tone and blood flow, increased adhesion of inflammatory cells to endothelium and loss of endothelial antithrombotic function [10]. It is known that the potent vasoconstrictor ET-1 may mediate homocysteine-induced endothelial damage and dysfunction [11].

2. Materials and methods

2.1. Materials

N-Acetylcysteine (NAC), PD98059, 2,7-dichlorofluorescin diacetate (DCF-DA) and homocysteine were purchased from Sigma Chemicals (St. Louis, MO). The pG3L3-basic vector, luciferase and β-galactosidase enzyme fluorescent kits were obtained from Promega (Madison, WI). Human ET-1 enzyme immunometric assay kit was from Assay Designs Inc. (Ann Arbor, MI). TRIzol and RT-PCR kit Human ET-1 enzyme immunometric assay kit was from Assay Designs Inc. (Ann Arbor, MI) based on a double-antibody sandwich technique [9]. 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 inter-assay and intra-assay coefficients of variation were below 3.3%.

2.4. RNA isolation and RT-PCR amplification

RNA was isolated from HUVECs with TRIzol (Invitrogen, Carlsbad, CA). Ultraviolet spectrophotometer reading with an A260/A280 ratio between 1.8 and 2.0 was deemed good RNA quality. First strand cDNA templates were created in final reaction conditions containing 2 μg of total RNA, 500 ng of oligo(dT), 10 mM dNTPs, 100 mM dithiothreitol and 50 units of superscript reverse transcriptase (Invitrogen). Primers were synthesized according to motif: CGTTGTTCGCTATGGACTTG (ET-1, sense); AGGCTATGGCTTCAGACAGG (ET-1, antisense); ACGGATTTGCTGATTTGG (GAPDH, sense) and TCCTGGAAGATGGATGAGG (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 for normalization of ET-1 mRNA in RT-PCR.

2.5. Western blot analysis

HUVECs with a density of 5 × 10^6 cells/ml were subjected to 50 μl cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 0.1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml aprotinin, 50 μg/ml leupeptin) for 30–40 min at 4 °C. The samples were centrifuged at 12,000 × g and supernatants were collected. The Bradford
method was used for protein measurement. A total of 10 μg of extracted protein was loaded to 10% SDS-polyacrylamide gel. Proteins were transferred to PVDF sequi-blot membranes (BioRad, Hercules, CA). Nonspecific binding sites were blocked for 2–3 h with 5% nonfat dried milk in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5). The membranes were incubated overnight at 4 °C with anti-ERK, anti-pERK monoclonal anti-mouse (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Jun/AP-1 polyclonal anti-rabbit (1:1000, Upstate and Chemicon, Temecula, CA) and the loading control anti-β-actin (1:1000, Santa Cruz Biotechnology) antibodies. After washing, the membranes were incubated for 2 h at room temperature with corresponding secondary antibodies. 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 intensity of immunoblot bands was detected.

2.6. Intracellular ROS production

The membrane-permeable probe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) (Molecular Probes, Eugene, OR) enters the cells and produces a fluorescent signal after intracellular oxidation by ROS such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical. Intracellular oxidant stress was monitored by changes in fluorescence intensity resulted from intracellular probe oxidation according to previously described method [10]. Following 24-h homocysteine treatment with or without α-ZAL and the ROS scavenger N-acetylcysteine (NAC, 10 μM) in 90% confluent HUVECs, cells were loaded with CM-H$_2$DCFDA (25 μM) for 50 min at 37 °C before being rinsed with the Hank’s solution three times. Cells were sampled randomly using a Nikon ECLIPSE TE2000-U fluorescence microscope (Tokyo, Japan). Fluorescent intensity was detected at the excitation wavelength of 488 nm and the emission wavelength of 510 nm.

2.7. Eukaryotic cell transfection luciferase assay

Based on structure characteristics of the target ET-1 DNA segment and the pGL3-basic vector, the nucleotide analysis software PRIME 5.0 was used for primer design. Two restricted endonuclease digestion sites including KpnI and Hind III were introduced at upstream 5′ and downstream 5′. The primer sequences were as the following: sense: −431 5′-GGGGTACCGAAAATGAAGCGAGCAA-3′; antisense: +135 5′-CCCAAGCTTAACGGGGAGAAAAGG-3′. HUVECs were transfected with 2 μg of the wild-type luciferase construct plasmid DNA using the lipofectin method (Invitrogen). To correct for variation in transfection efficiency, pSV-β-galactosidase plasmid DNA (β-gal, 1 μg) was cotransfected. Following transfection with various agents, the ratio between luciferase and β-gal activities was used as the relative luciferase activity.

2.8. Data analysis

For each experimental series, data are presented as mean ± S.E.M. Statistical significance (p < 0.05) for each variable was estimated by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test for post hoc analysis.

3. Result

3.1. Effects of α-ZAL on homocysteine-induced ET-1 production

ET-1 plays an essential role in the pathogenesis of endothelial dysfunction and atherosclerosis [7]. To explore the possible mechanism of action underneath α-ZAL-induced cardiovascular protection, we examined the effect of α-ZAL on homocysteine-induced ET-1 release. Results in Fig. 1 demonstrated that α-ZAL (1–1000 nM) effectively inhibited homocysteine (500 μM)-induced ET-1 production. The concentration of 0.01 μM (10 nM) was chosen for α-ZAL in our remaining experiments.

3.2. Effect of α-ZAL on homocysteine-induced ROS accumulation

Result shown in Fig. 2 indicates that intracellular ROS was significantly enhanced following a 24-h incubation with homocysteine (500 μM). Coincubation of homo-

![Fig. 1. Effect of α-ZAL (1–1000 nM) on homocysteine (HCY, 500 μM)-induced ET-1 production in HUVECs. HUVECs were incubated with various concentrations of α-ZAL in the presence of HCY for 24 h. Mean ± S.E.M., n = 3, *p < 0.05 vs. control, #p < 0.05 vs. HCY.](image-url)
cysteine with α-ZAL (0.01 μM) or the ROS scavenger N-acetylcysteine (NAC, 10 μM) effectively nullified homocysteine-induced ROS accumulation. Neither α-ZAL nor NAC affected ROS accumulation by itself.

3.3. Effect of α-ZAL on homocysteine-induced ERK activation

Western blot analysis revealed that ERK phosphorylation (pERK/ERK ratio) was significantly enhanced following a 24-h incubation of homocysteine (500 μM). Interestingly, coincubation of homocysteine with α-ZAL (0.01 μM), the ERK inhibitor PD98059 (30 μM), or the ROS scavenger NAC (10 μM) abrogated homocysteine-induced ERK phosphorylation (pERK/ERK ratio) in HUVECs (Fig. 3). Total ERK protein expression was not affected by homocysteine, α-ZAL, PD98059 or NAC treatment (data not shown).

3.4. Effect of α-ZAL on homocysteine-induced ET-1 secretion and gene expression, transcription factor c-Jun/AP-1 and transcriptional activity of the ET-1 gene codon region reporter gene AP-1

Fig. 4A exhibits that a 24-h incubation of homocysteine (500 μM) promoted ET-1 secretion in HUVECs,
which was blunted by either α-ZAL (0.01 μM) or the ROS scavenger NAC (10 μM). Data depicted in Fig. 4B indicated that a 24-h incubation of homocysteine (500 μM) upregulated ET-1 mRNA expression, which was blocked by either α-ZAL (0.01 μM) or NAC (10 μM). Neither α-ZAL nor NAC affected ET-1 secretion and ET-1 gene expression by itself. Western blot analysis revealed that α-ZAL (0.01 μM) or NAC (10 μM) effectively antagonized homocysteine (500 μM)-induced upregulation of c-Jun/AP-1 protein (Fig. 5A). Transient transfection assay indicated that homocysteine (500 μM, 24-h incubation) enhanced transcriptional activity of the ET-1 gene codon region reporter gene AP-1 in HUVECs, which was blocked by either α-ZAL (0.01 μM) or NAC (10 μM) (Fig. 5B). Similarly, neither α-ZAL nor NAC affected AP-1 transcriptional activity by itself.

4. Discussion

Since the postulation of a homocysteine theory for atherosclerosis pathogenesis [12], ample of clinical and experimental evidence has consolidated the notion of hyperhomocysteinemia as an independent risk factor for the development of atherosclerosis via impaired endothelial function. Homocysteine is a sulfur-containing amino acid formed primarily from methionine by demethylation with l-homocysteine being the primary active form in a variety of tissues or cells. It triggers accumulation of intracellular ROS including H₂O₂, O₂⁻ and -OH through sulfur auto-oxidation, leading to cell injury and endothelial dysfunction [13]. Sustained exposure of endothelial cells to high levels of homocysteine may produce S-nitroso-homocysteine complex, which suppresses the bioavailability of nitric oxide [13,14]. Elevated plasma homocysteine levels have been demonstrated to contribute to endothelial dysfunction in a wide variety of vascular beds including impaired
endothelium-dependent function, increased recruitment and adhesion of inflammatory cells to endothelium, and loss of endothelial anti-thrombotic function [13]. It has been postulated that homocysteine-induced endothelial dysfunction may serve as a key predictor for ischemic heart disease, renal failure, metabolic syndrome, infarct, postmenopausal syndrome and senile diseases including Alzheimer’s disease [13,15].

As a natural occurring phytoestrogen, α-ZAL is comparable to estrogen with regard to its anti-atherogenic effect with much smaller effect on uterine and mammary gland growth [16]. Therefore, α-ZAL may be considered as a promising replacement for estrogen with little undesired effect [6,16]. Treatment of atherosclerosis using phytoestrogens has drawn great attention recently. Several hypotheses have been postulated for their mechanisms of action against atherosclerotic development including alleviated LDL oxidation and scavenged oxidative free radicals. Cheng and coworkers reported that phytoestrogen resveratrol may inhibit ET-1 gene expression and secretion of ET-1 [19]. ROS acts as a codon transcriptional factor AP-1, which in turn elicits additional phosphorylation of ERK via ROS accumulation and further activates the transcriptional factor AP-1, which in turn elicits additional expression and secretion of ET-1 [19]. ROS acts as a codon or promoter in this process. The fact that the antioxidant NAC blocked the expression and secretion of ET-1 further testified the role of ROS. Our data favor the sequential event of ROS, activation of protein kinase (ERK) and induction of the nuclear transcription factor AP-1 as a classical pathway for ET-1 gene expression. Our data suggested possible interaction between α-ZAL and the ET-1 signal cascade, suggesting that α-ZAL may inhibit ERK phosphorylation through suppressing oxidative oxygen molecules. Our observation revealed that inhibition of ERK phosphorylation is capable of preventing AP-1 activation, thus suppressing ET-1 gene codon, ET-1 mRNA and protein expression, ultimately secretion of the peptide. These data elucidated that α-ZAL may negatively regulate the ET-1 signaling system through alleviating ROS production and ERK phosphorylation in endothelial cells. However, it should be mentioned that phosphorylation of ERK may promote both cell survival and cell death in a broad range of cellular activities and physiological processes [20]. Thus, the protective effects of α-ZAL may not necessarily be mediated through inhibition of ERK phosphorylation, which can itself be detrimental. Other signaling pathways may exist and contribute to the α-ZAL-induced beneficial effect on endothelial function including inhibition on the Ras-Raf-ERK-AP-1-ET-1 pathway. Further study is warranted to scrutinize the rather complicated signaling pathways and interaction with ROS in α-ZAL-elicited vascular and endothelial protection.

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