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Safrole oxide induced human umbilical vein vascular endothelial cell differentiation into neuron-like cells by depressing the reactive oxygen species level at the low concentration

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Abstract

Previously, we found that 5–25 μg/ml safrole oxide could inhibit apoptosis and dramatically make a morphological change in human umbilical vein vascular endothelial cells (HUVECs). But the possible mechanism by which safrole oxide function is unknown. To answer this question, in this study, we first investigated the effects of it on the activity of nitric oxide synthetase (NOS), the expressions of Fas and integrin β4, which play important roles in HUVEC growth and apoptosis, respectively. The results showed that, at the low concentration (10 μg/ml), safrole oxide had no effects on NOS activity and the expressions of Fas and integrin β4. Then, we investigated whether HUVECs underwent differentiation. We examined the expressions of neuron-specific enolase (NSE) and neurofilament-L (NF-L). Furthermore, we analyzed the changes of intracellular reactive oxygen species (ROS). After 10 h of treatment with 10 μg/ml safrole oxide, some HUVECs became neuron-like cells in morphology, and intensively displayed positive NSE and NF-L. Simultaneously, ROS levels dramatically decreased during HUVECs differentiation towards neuron-like cells. At the low concentration, safrole oxide induced HUVECs differentiation into neuron-like cells. Furthermore, our data suggested that safrole oxide might perform this function by depressing intracellular ROS levels instead of by affecting cell growth or apoptosis signal pathways.

Keywords: Safrole oxide; HUVEC; Differentiation; Neuron

1. Introduction

Vascular endothelial cells (VEC) have very important roles in vascular development and in maintaining the normal functions of blood vessels. The coordinated regulation of vascular endothelial cell growth, apoptosis, and differentiation is essential for the normal functions of VEC. There are growth signal pathways, apoptosis signal transduction pathways, and differentiation signal pathways in VECs. It has been reported that targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis [1]. The results of this report showed that blockade of growth signaling pathways in VECs could lead to vascular defects during embryonic development due to increased endothelial cell apoptosis, significantly, endothelial cell proliferation and differentiation were not affected [1]. The data suggested that when the VEGF-mediated growth signaling pathways were impaired, VECs could select apoptosis or differentiation during embryonal development.

It has been shown that FGFs also play important roles in embryonic development, angiogenesis, wound healing and

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some malignancies. It was reported that bFGF was a much stronger mitogen for VECs than VEGF in vitro [2,3]. Thus, controlling VEC growth stimulated by bFGF is a promising new approach in vascular development research and cancer therapy [4]. We have been studying the growth mediated by bFGF and the apoptosis induced by deprivation of bFGF in HUVECs. Our previous experiment results showed that integrin β4 was a key membrane protein in HUVEC apoptosis induced by deprived of bFGF [5–8]. Apoptosis of VEC is likely regulated by survival and death signals. Among the survival signals, NOS is an important element [9]. Activation of the endothelial NOS, leads to enhanced synthesis of NO, which promotes HUVEC survival. Inhibition of the survival signaling pathways mediated by NO caused massive HUVEC apoptosis [10]. Recently, we found a small molecule, safrole oxide, could trigger HUVEC apoptosis by upregulating of Fas, integrin β4 and depressing of ROS level at high concentrations [11]. These data indicate that NOS, Fas and integrin β4 are important elements and may have some relations in controlling HUVEC growth and apoptosis.

On the other hand, we observed that, when HUVECs were exposed to safrole oxide of low concentrations (5–25 μg/ml), the apoptosis induced by deprivation of bFGF was inhibited and some cells became neuron-like cells in morphology [12]. This finding initiated us to study the possible mechanism by which safrole oxide acts in HUVECs at the low concentration. To our knowledge, it is the first report that, at the low concentration, safrole oxide could induce HUVECs differentiation into neuron-like cells by depressing intracellular ROS levels instead of by affecting cell growth or apoptosis signal pathways.

2. Materials and methods

2.1. Reagents

Safrole oxide [3,4-(methylenedioxy)-1-(2′,3′-epoxypropyl)-benzene] was synthesized by the reaction of safrole with 3-chloroperoxybenzoic acid and purified by silica gel column chromatography [13]. It was dissolved in ethanol and applied to cells such that the final concentration of ethanol in the culture medium was below 0.01% (vol/vol). Ethanol at a concentration of 0.1% (vol/vol) did not affect the viability of the cells. M199 medium was purchased from Gibco BRL Co., Grand Island, NY. DCHF (2′,7′-dichlorofluorescin) was purchased from Sigma, Co. USA. Fetal bovine serum (FBS) was purchased from Hyclone Lab Inc. USA. Basic fibroblast growth factor (bFGF) was extracted from bovine brains by the method of Lobb and Fett [14] in our laboratory. Primary antibodies (Rabbit anti-rat neuron-specific enolase (NSE), neurofilament-L (NF-L) and Mouse anti-human integrin β4) and secondary antibody (FITC- IgG) were purchased from Santa Cruz Co. Nitric oxide synthase (NOS) detection kit was purchased from Nanjing Jiancheng Co. All other reagents were ultrapure grade.

2.2. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were obtained in our laboratory by using the method of Jaffe et al. [15]. The cells were cultured on gelatin-coated plastic dishes with M199 medium that was supplemented with 20% FBS and bFGF 70 mg/ml at 37 °C in 5% CO_2/95% air (normal group). All experiments were performed on the cells from 10 to 20 passages.

2.3. Exposure of HUVECs to safrole oxide

HUVECs were washed once with the medium and divided into two groups when the cultures of HUVECs reached sub-confluence: in control group, cells were cultured in the medium without bFGF and serum; in safrole oxide-treated group, the cells deprived of bFGF and serum were treated with 10 μg/ml safrole oxide for 10 h, 24 h and 48 h. The morphological changes of the cells were observed under phase contrast microscope (Nikon, Japan).

2.4. Cell viability analysis

Cells were seeded into 96-well plates and divided into three groups: in normal group, cells were cultured in the supplemented medium with bFGF and serum; in control group, cells were cultured in the medium without bFGF and serum; in safrole oxide-treated group, the cells deprived of bFGF and serum were treated with 5 μg/ml, 10 μg/ml and 25 μg/ml safrole oxide, respectively for 24 h. Cell growth was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (Sigma Chemical Co. USA) assay. After cells were treated with safrole oxide 6 h, 20 μl of MTT (5 mg/ml) in PBS solution was added to each well and then the plate was further incubated for 4 h. All remaining supernatant were removed and 100 μl of DMSO was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 min of incubation to ensure all crystals were dissolved, the light absorption was measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI co., USA).

2.5. Immunofluorescence assay

Immunofluorescence was performed on subconfluence cells after treatment for 10 h with safrole oxide to examine the expression of integrin β4. The cells were washed with 0.1M phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min. After washing with 0.1 M PBS, they were incubated with 30% H_2O_2/methyl (1/9 vol/vol) for 5 min and blocked with sheep serum diluted at 1:50 in 0.1 M PBS for 20 min at room temperature. Then, primary antibodies (mouse anti-human integrin β4) were added and incubated in a humid chamber over night at 4 °C. PBS, 0.1 M, replaced primary antibodies as negative control. After washing with 0.1 M PBS, secondary antibodies (goat anti-mouse) were added and incubated for 20 min at 37 °C. After washing with 0.1M PBS, samples were evaluated with laser scanning confocal microscopy (Leica, Germany). The immunofluorescence techniques allow semiquantitative evaluation of protein expression [16].

2.6. Western immunoblot analysis

Cells were cultured in the absence or presence of safrole oxide for 10 h. The total protein of the cells was prepared as described by Lipscomb E.A. [17]. The concentration of each protein lysate was determined by the Bradford Protein Assay [18]. Equal amount of total protein was loaded on 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose transfer membrane (Amersham Pharmacia Biotech., USA). After blocking with 5% skim milk in PBS, 0.5% (vol/vol) Tween 20 for 1 h, the membrane was incubated with monoclonal Fas protein antibody (mouse anti human) overnight at 4 °C, then incubated with HRP-linked secondary antibodies (goat anti mouse) for 1 h at room temperature (RT), followed by color development with 0.06% DAB (diaminobenzidine) and 0.03% H202 in PBS for 3–5 min, at RT. Distilled water was used to cease the reaction. Monoclonal anti-β-actin antibody (mouse anti human) was used to ascertain that equal amount of protein was loaded. The relative quantity of protein was analyzed by Imagetool software.

2.7. NOS activity assay

For the preparation of enzymes, after harvested and washed, the cells (1×10^6) were homogenized with ultrasonic (400W, 12 min) in 3 ml buffer A (20 mM Tris–HCl, pH 7.0, 10 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.34 mM sucrose) on ice. After centrifuged at 800 rpm, 4 °C for 15 min, the supernatant was centrifuged again at 100,000 rpm, 4 °C for 1 h, then, was used for NOS activity assay.