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FUCOSTEROL DECREASES ANGIOTENSIN CONVERTING ENZYME LEVELS WITH REDUCTION OF GLUCOCORTICOID RECEPTORS IN ENDOTHELIAL CELLS

Hiromi Hagiwara*, Ken-ichi Wakita, Yuji Inada, and Shigehisa Hirose

Department of Chemistry, Tokyo Institute of Technology,
Ookayama, Meguroku, Tokyo 152, Japan

*Faculty of Pharmaceutical Science,
Showa University, Hatanodai, Shinagawaku, Tokyo 142, Japan

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The modulation of angiotensin converting enzyme(ACE) levels was studied using fucosterol, one of phytosterols, in cultured bovine carotid endothelial cells. Addition of fucosterol to the culture medium resulted in the decrease of ACE activity of endothelial cells; however, fucosterol did not directly inhibit ACE activity. Dexamethasone elevated the levels of ACE in normal cells, but this effect was not seen in the fucosterol-treated cells. Receptor assays showed that the amount of glucocorticoid receptors in fucosterol-treated cells decreased to an undetectable level. These results indicate that fucosterol lowers the ACE levels on the endothelial cells by inhibiting the synthesis of glucocorticoid receptors involved in the regulation of ACE levels.

Angiotensin converting enzyme(ACE) is a membrane-bound dipeptidyl carboxypeptidase located mainly on the endothelial cells(1,2). It converts the decapeptide angiotensin I to the octapeptide angiotensin II, a powerful vasoconstrictor. The enzyme, having a relatively broad specificity, is also involved in the degradation of bradykinin, a vasodilator, and numerous other peptides. The levels of ACE expressed on the endothelial cells are susceptible to glucocorticoid modulation(3).

Fucosterol was first isolated in 1934 from brown algae(4). Its physiological functions in human body after ingestion are not clear. We previously reported that phytosterols such as fucosterol and sitosterol enhance the plasminogen activator production in endothelial cells(5,6). In the present study, we examined the effects of phytosterols on ACE levels using cultured endothelial cells and found that fucosterol lowers the ACE levels. Since glucocorticoid-induced alterations in ACE levels have already been recognized, we first reasoned that fucosterol may act as an

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antagonist of glucocorticoid and directly modulates the ACE levels; however, quite unexpectedly, receptor assays revealed that fucosterol does not compete with glucocorticoid for the binding sites. Rather, it inhibited the production of glucocorticoid receptors in the endothelial cells. These unique properties of fucosterol would make it an agent of pharmacological interest as well as a valuable aid to study the regulation of endothelial cell functions including productions of plasminogen activator, ACE, and glucocorticoid receptors.

MATERIALS AND METHODS

Fucosterol and sitosterol were supplied from Kimitsu Chemical Industry, Japan and Tama Biochemical Co., Ltd., Japan, respectively. Dexamethasone was purchased from SIGMA Chemical Company, St. Louis, Mo, USA. [1,2,4,6,7-^{3}H] Dexamethasone was obtained from Amersham, U.K. Bz-Gly-His-Leu·H_2O and His-Leu were purchased from Protein Research Foundation, Osaka, Japan and o-phthalaldehyde was purchased from Kanto Chemical Co., Inc., Tokyo, Japan.

Endothelial Cell Culture—Endothelial cells obtained from bovine carotid artery were cultured by the method previously described(5). The cells which had been passaged 14-18 times were used in the present study.

Assay of Angiotensin Converting Enzyme Activity—ACE activity was measured by a modification of the fluorimetric method(7). Endothelial cells were subcultured in petri dishes(22 cm^2) with culture medium containing various concentrations of fucosterol in ethanol(final concentration 1%). This concentration of ethanol did not affect ACE activity. After visual confluency, the monolayers were washed with cold serum-free medium. The washed monolayers were incubated, at 37°C, with 1.5ml of reaction mixture containing 5mM Bz-Gly-His-Leu(synthetic substrate), 150mM NaCl and 50mM Tris-HCl(pH 7.9) in the presence and the absence of 1 μM captopril, an ACE inhibitor. At a given time, a 100μl of sample was removed from the reaction mixture and added to 1.4ml of 0.5M NaOH to stop the reaction. The product His-Leu was detected by adding 100μl of 10mg/ml o-phthalaldehyde in ethanol, incubating at 25°C for 5min and adding 250μl of 6N HCl. The fluorescence of the mixture was measured with emission at 495nm and with excitation at 365nm. The standard curve was obtained using known concentration of His-Leu. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per hour per 10^6 cells.

Cytosol Preparation—Endothelial monolayers cultured in culture medium with the presence and the absence of 25μM fucosterol for 48hr were washed with cold saline and were removed from plastic petri dishes(55 cm^2) with a rubber policeman. The cells were washed with cold phosphate buffer(pH 7.8) containing 30mM sodium phosphate, 60mM NaCl, 3mM $MgCl_2$, 1mM EDTA, 10mM 2-mercaptoethanol, 0.25mM PMSF, 12μM leupeptin, 7.3μM pepstatin, 10% glycerol and 10mM sodium molybdate. The cells were homogenized with a Teflon homogenizer. Cytosol was prepared by centrifugation at 110,000 x g for 60min at 4°C.

Binding Assay of Glucocorticoid Receptor—Competition assays of steroid binding were carried out in the phosphate buffer(pH 7.8) at 4°C. Cytosol obtained from approximately $2x10^7$ cells was incubated for 24hr with 20nM [^{3}H]dexamethasone(0.5μCi) in the presence and the absence of radioinert steroid. Complexes of [^{3}H]dexamethasone and glucocorticoid receptor were separated from free [^{3}H]dexamethasone by gel filtration using

Bio-Gel P-6DG column(0.7 x 10cm). Radioactivity of fractions containing the complexes was measured with Aloka LSC-700 Liquid scintillation counter using ATOMLIGHT obtained from DUPONT as scintillant.

RESULTS AND DISCUSSION

ACE activity of cultured bovine carotid endothelial cells was estimated to be 25 nmoles/hr/10^6 cells by the fluorimetric method using Bz-Gly-His-Leu as synthetic substrate. This activity was completely inhibited by 1μM captopril, a specific inhibitor of ACE. Using these cells, we examined the effects of fucosterol and sitosterol on the ACE levels. Addition of 25μM fucosterol to culture medium resulted in a gradual loss of the ACE activity on the cell surface(Fig. 1). Although structurally very similar, sitosterol had no effect, indicating the importance of the double bond at the side chain(Fig. 1). This inhibitory effect of fucosterol was not due to direct interactions between fucosterol and ACE since addition of 25μM fucosterol to reaction mixture during incubation period did not affect the ACE activity at all.

Dexamethasone, a synthetic analog of glucocorticoid, elevated the levels of ACE in normal cells, as already reported by others(3,8,9). We then examined the combined effects of dexamethasone and fucosterol. As shown in Fig. 2, fucosterol lowered the dexamethasone-stimulated ACE levels

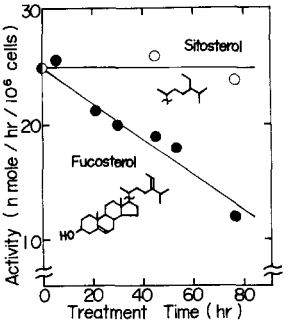

Fig. 1. ACE Activity of Endothelial Cells Treated with Fucosterol or Sitosterol for Various Length of Time. Endothelial cells were subcultured with culture medium supplemented with 25μM fucosterol or 25μM sitosterol for several periods. Endothelial monolayers prepared were washed with serum-free medium and were incubated with reaction mixture for 1hr. The activity was measured by the method described in MATERIALS AND METHODS.

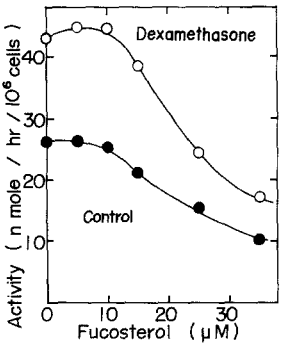

Fig. 2. Relation between ACE Activity on Dexamethasone-Treated Cells or Untreated Cells and Amount of Fucosterol Added. Endothelial cells were subcultured in culture medium supplemented with fucosterol alone or fucosterol plus 1μM dexamethasone for 72hr. Endothelial monolayers were washed with serum-free medium and incubated with reaction mixtures containing the ACE substrate Bz-Gly-His-Leu for 1hr. The ACE activity was measurerd as described in MATERIALS AND METHODS. Even at 25 μM fucosterol, morphological changes of the cells were not observed.

as well as those of control cells in a dose-dependent manner. To investigate by what mechanisms fucosterol alters the ACE levels on the endothelial cells, we explored the following two possibilities: 1) fucosterol may act as an antagonist of glucocorticoid or 2) fucosterol may somehow interfere with the production of glucocorticoid receptors responsible for the maintenance of ACE levels. Radioreceptor assays using [^{3}H]dexamethasone indicated that fucosterol reduces the number of glucocorticoid receptors available for agonist binding by inhibiting the receptor biosynthesis rather than by competing with glucocorticoid for the binding sites(table I).

Table I. Reduction of Glucocorticoid Receptor Levels in Cultured Bovine Endothelial Cells by Fucosterol

Source of Receptors	Competitor	Radioactivity(cpm)
untreated cells	none	2067
	fucosterol	2267
	dexamethasone	569
fucosterol-treated cells	none	677

Cytosols (200 μl) were prepared from normal and fucosterol-treated cells (48 hr-treatment), and incubated with 20nM [^{3}H] dexamethasone in the presence and the absence of 10μM dexamethasone or 10μM fucosterol for 24 hr at 4° C. The radioligand-receptor complexes formed were measured by the method described in MATERIALS AND METHODS.

In summary, using the same cell cultures employed previously to demonstrate that fucosterol markedly stimulates the production of plasminogen activator, we demonstrated here that fucosterol can also influence the biosynthesis of ACE. In experiments designed to unravel the mode of action of fucosterol, we showed that treatment of the endothelial cells with fucosterol results in the decrease in glucocorticoid receptor levels, which in turn leads to lowered ACE levels. These results suggest that glucocorticoid and its receptor play an essential role for maintaining proper levels of ACE on the endothelial cells and that fucosterol could be used also in a variety of other cells to examine how the absence of glucocorticoid receptors affects their functions.

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