VOL. 52, NO. 2, FEB. 1999 THE JOURNAL OF ANTIBIOTICS pp. 171 — 174

NOTES

11-Keto-9(*E*),12(*E*)-octadecadienoic Acid, a Novel Fatty Acid that Enhances Fibrinolytic Activity of Endothelial Cells

CHIKARA SHINOHARA, KEIJI HASUMI*, TOSHIHIRO CHIKANISHI, TADASHI KIKUCHI and AKIRA ENDO

Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183-8509, Japan

(Received for publication October 16, 1998)

Vascular endothelial cells play an important role in the regulation of fibrinolysis in the blood vessel by producing plasminogen activators and plasminogen activator inhibitors. Defects in physiological regulation of the balance of the activities between plasminogen activator and the inhibitors may cause vascular diseases such as thromboembolism and atherosclerosis. In patients with such diseases, the inhibitor may be dominant in this balance. Actually, the expression of plasminogen activator inhibitor-1 is increased in atherosclerotic arteries[1~3], and elevated levels of the inhibitor are suggested to be a risk factor for thrombotic disease[4~6]. The present investigation was undertaken to identify agents that can enhance fibrinolytic activity of vascular endothelial cells. A search from microbial cultures has lead to the isolation of a novel fatty acid, 11-keto-9(*E*),12(*E*)-octadecadienoic acid (KOD), as an active compound (Fig. 1). In this paper, we describe the fermentation production, isolation, and structure elucidation of KOD.

The producing strain, which was isolated from a soil sample collected in Hiroshima, Japan, was identified to be *Trichoderma* sp. F5594 from the following observations: the growth on potato glucose agar was rapid at 25°C, and the diameter of the colony reached 100 mm on day 5; the surface of the colony was dark green and the reverse white with a yellowish center; phialophores (approximately 5 μm in width) were hyaline to light yellowish green, septated, arose simply from the hyphae, and branched verticillately; one to several ampulliformed phialides (2~3 μm × 5~15 μm) were formed at the tip of the phialophore; phialospores (2~3 μm in diameter) were green, subglobose to ovoid, smooth surfaced, and formed a spherical conidial head at the apex of the phialide.

For production of KOD, the strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of 3.5% glucose, 1.0% corn starch, 2.0% soybean meal, 0.5% peptone, 0.5% meat extract, 0.3% yeast extract, 0.2% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4$, and 0.01% CB442 (antifoam; Nippon Oil and Fat Co., Tokyo, Japan). The flask was incubated at 25°C for 3 days on a rotary shaker (180 rpm). One ml of the culture was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the same medium, and the flasks were incubated at 25°C on a rotary shaker (180 rpm). The production of KOD reached a maximum (40 μg/ml) on day 7. The mycelium was obtained by filtering the combined cultured broth (5.0 liters) and was extracted three times with 1 liter of acetone. After concentration to remove the organic solvent, the acetone extracts were combined with the culture filtrate. The pooled solution was adjusted to pH 3 with HCl and extracted with ethyl acetate (once with 5 liters and twice with 2.5 liters). The combined organic layer was dried over sodium sulfate and concentrated to dryness, giving 2.9 g of an oily residue, which was then applied to a silica gel column (40 × 180 mm). After washing with *n*-hexane (3 liters), the column was developed with *n*-hexane/acetone (9 : 1, 3 liters). The active fractions were combined and concentrated to dryness, yielding 0.78 g of a residue. The residue was further purified by HPLC on an Inertsil PREP-ODS column (20 × 250 mm; GL Sciences, Tokyo,

Fig. 1. 11-Keto-9(*E*),12(*E*)-octadecadienoic acid (KOD).

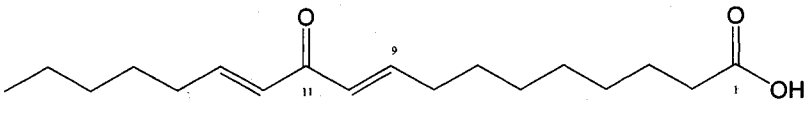


Table 1. Physico-chemical properties of KOD.

Nature	Light brown oil
Molecular formula	$C_{18}H_{30}O_3$
FAB-MS (*m/z*)	295 $(M+H)^+$, 317 $(M+Na)^+$, 333 $(M+K)^+$
HRFAB-MS (*m/z*)	
Found:	295.2270 $(M+H)^+$
Calcd:	295.2273 (for $C_{18}H_{31}O_3$)
UV λ_{max} (MeOH) nm (ε)	253 (10,100)
IR ν_{max} (KBr) cm^{-1}	2930, 2865, 1731, 1707, 1665, 1633, 1613, 1453, 979, 802, 724, 460
^1H NMR ($CDCl_3$)	δ 6.94 (1 H, dt, J= 6.9, 15.8), 6.87 (1 H, dt, J= 6.9, 15.8), 6.31 (2 H, d, J= 15.8), 2.34 (2 H, t, J= 7.3), 2.28 (4 H, m), 1.63 (2 H, m), 1.47 (4 H, m), ~1.3 (10 H, m), 0.89 (3 H, t, J= 6.0)
^{13}C NMR ($CDCl_3$)	δ 189.76, 178.96, 148.18, 147.89, 128.69, 128.64, 33.38, 32.64, 32.61, 31.38, 28.99, 28.99, 28.91, 28.08, 27.85, 24.62, 22.45, 13.98

Japan) developed with acetonitrile/0.1% H_3PO_4 (52:48) at a rate of 25 ml/minute at 40°C. The active fractions were concentrated and extracted with ethyl acetate. The organic extracts were dried over sodium sulfate and concentrated to dryness to give 16 mg of purified active principle.

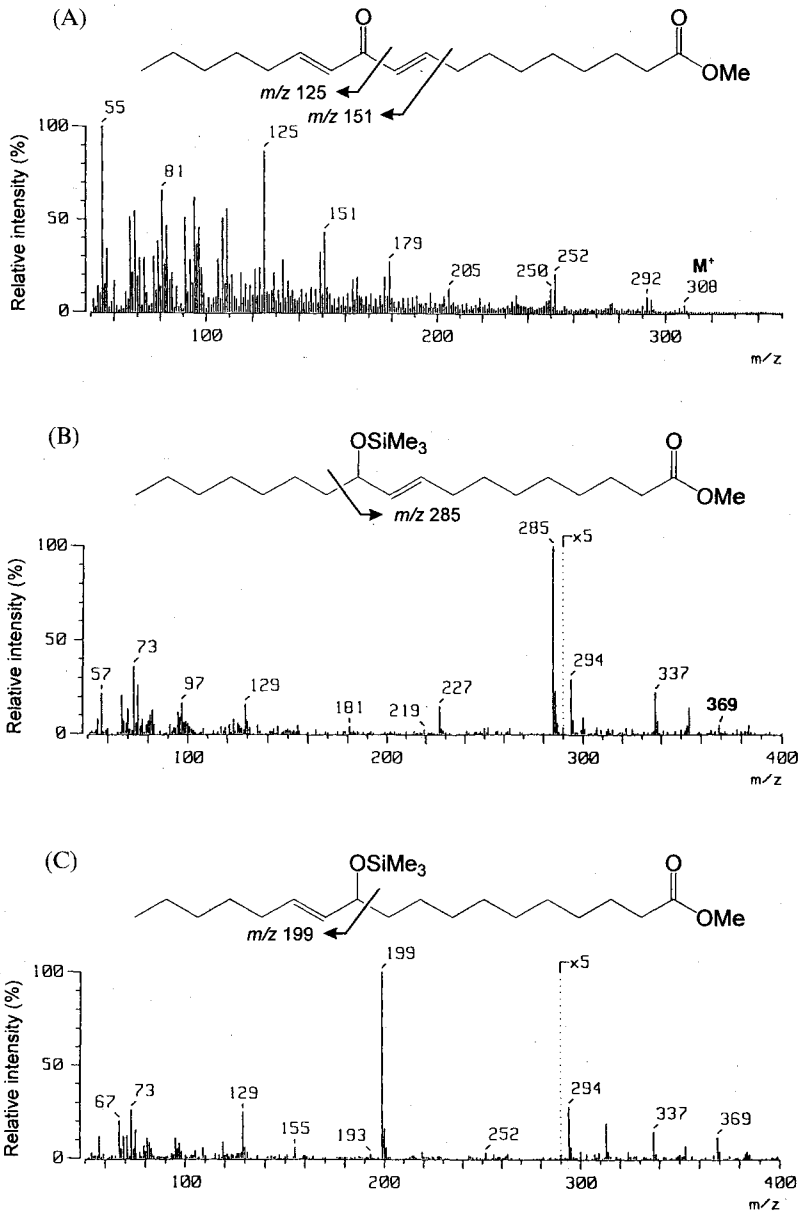
The physico-chemical properties of the active principle are summarized in Table 1. The molecular formula was established to be $C_{18}H_{30}O_3$ from the HRFAB-MS spectrum. The NMR analyses revealed the presence of a methyl, eleven aliphatic methylenes, four olefinic methines, and two carbonyl carbons. The ^1H-^1H COSY and HMBC spectra suggested that the compound possessed the following partial structures: $-(CH_2)_m$-COOH, $-(CH_2)_nCH_3$, and $-(CH_2)_xCH=CHCOCH=CH(CH_2)_y-$, where m, n, x and y represent indefinite numbers. To determine the number of methylene unit that connect the partial structures, derivatives of the compound were prepared and analyzed by mass spectrometry. The methyl ester derivative yielded prominent fragment ion peaks at *m/z* 125 $[(CH_3(CH_2)_4CH=CHCO)^+]$ and 151 $[(CH_3(CH_2)_4CH=CHCOCH=CH)^+]$ (Fig. 2). Two trimethylsilyl derivatives of partially reduced methyl KOD gave prominent ion peaks at *m/z* 285 $[Me_3SiO^+=CHCH=CH(CH_2)_7COOCH_3$, base peak] and *m/z* 199 $[Me_3SiO^+=CHCH=CH(CH_2)_4-CH_3$, base peak], respectively (Fig. 2). These results demonstrated that the compound had Δ^9 and Δ^{12} double bonds. The configuration at both double bonds could be assigned to be *E* according to the following observations: coupling constants between 9-H and 10-H and between 12-H and 13-H were relatively large (15.8 Hz for both) in the ^1H NMR spectrum; an absorption band at 979 cm^{-1} was present in the IR spectrum. These observations established that the active principle was 11-keto-9(*E*),12(*E*)-octadecadienoic acid (Fig. 1).

The fibrinolytic activity of cultured bovine aortic endothelial cells was elevated by 1.5- to 4-fold after treatment with KOD at concentrations ranging from 50 to 650 μM. The concentration required for 2-fold elevation of the activity was approximately 250 μM.

The red alga *Lithothamnion corallioides* is reported to convert linoleic acid into the oxidation product 11-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid, which is further oxidized enzymatically to yield 11-keto-9(*Z*),12(*Z*)-octadecadienoic acid, a geometrical isomer of KOD, as a minor product[7]. Although the enzymatic conversion of linoleic acid into the hydroxyoctadecadienoic acid is oxygen-dependent, neither lipoxygenase nor cytochrome P-450 is involved in the conversion, and the hydroxyl oxygen is postulated to be derived from water but not from molecular oxygen[7]. It would be of interest to examine whether similar mechanism is involved in the formation of KOD by *Trichoderma* sp. F5594.

Elevation of fibrinolytic activity in vascular endothelial cells could be a useful approach in strategies aimed at the prevention of vascular diseases such as thromboembolic diseases and atherosclerosis. KOD is a simple

Fig. 2. EI-MS spectra of methyl KOD (A) and trimethylsilyl derivatives of partially reduced KOD (B and C).



molecule, and thus, it would be useful as a template for designing effective modulators of endothelial fibrinolytic activity.

Experimental

Spectroscopic Analyses

The UV spectrum was measured in MeOH on a model 320 spectrometer (Hitachi, Tokyo, Japan) and the IR spectrum on an IR-810 spectrometer (JASCO, Tokyo, Japan) with KBr. The NMR spectra were measured in $CDCl_3$ on an EX-270 spectrometer (JEOL) at 270 MHz for ^1H and 68 MHz for ^{13}C. The mass spectrum was taken on an SX-102A spectrometer (JEOL, Tokyo, Japan): for FAB-MS spectroscopy, glycerol was used as a matrix. GC-MS was performed using a capillary column (CP-Sil 88, 0.25 mm × 50 m, GL Sciences, Tokyo, Japan). The column temperature was kept at 50°C for 1 minute after injection of a sample and then increased at a rate of 32°C/minute up to 150°C, subsequently at a rate of 4°C/minute up to 225°C.

Methyl KOD and Trimethylsilyl Derivatives of Partially Reduced Methyl KOD

Methyl KOD was prepared using diazomethane. The methyl ester derivative (0.25 mg) was partially reduced by sodium borohydride (30 mg) in 3 ml methanol at 22°C for 30 minutes. The resulting alcohols, methyl 11-hydroxy-9-octadecenoate and methyl 11-hydroxy 12-octadecenoate, were trimethylsililated in 100 μl of a mixture of pyridine and bis(trimethylsilyl)-trifluoroacetamide at 25°C for 30 minutes[7].

Determination of Fibrinolytic Activity of Vascular Endothelial Cells

Endothelial cells were isolated from bovine aorta and subcultured for approximately 6 passages in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 100 units/ml benzylpenicillin and 100 μg/ml streptomycin sulfate. For assays, cells were seeded into 96-well tissue culture plates (5×10^5 cells/50 μl per well) and grown at 37°C for 24 hours. Subsequently, cells were washed twice with medium A (the above medium devoid of $NaHCO_3$ but supplemented with 20 mM HEPES, pH 7.4) and preincubated at 37°C for 6 hours in 50 μl of medium A with or without KOD. At the end of the incubation, cells were washed with ice-cold buffer A (50 mM Tris-HCl and 100 mM NaCl, pH 7.4) and then received 100 μl of buffer A containing 0.1 μM plasminogen and 0.1 mM S-2251 (H-D-valyl-leucyl-lysine-*p*-nitroanilide). After incubation at 37°C for 4 hours, the release of *p*-nitroaniline was determined by measuring the change in absorbance at 405 nm.

Acknowledgments

We thank AKIKO HASHIMOTO and AKINORI HAMANAKA for technical assistance. This work was supported in part by grants (to K.H.) from the Ministry of Health and Welfare, Japan and the Ministry of Education, Science, Sports and Culture, Japan.

References

1) AZNAR, J. & A. ESTELLES: Role of plasminogen activator inhibitor type 1 in the pathogenesis of coronary artery diseases. Haemostasis 24: 243~251, 1994
2) LUPU, F.; G. E. BERGONZELLI, D. A. HEIM, E. COUSIN, C. Y. GENTON, F. BACHMANN & E. K. O. KRUITHOF: Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. Arterioscler. Thromb. 13: 1090~1100, 1993
3) SCHNEIDERMAN, J.; M. S. SAWDEY, M. R. KEETON, G. M. BORDIN, E. F. BERNSTEIN, R. B. DILLEY & D.J. LOSKUTOFF: Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. Proc. Natl. Acad. Sci. USA 89: 6998~7002, 1992
4) ERICKSON, L. A.; G. J. FICI, J. E. LUND, T. P. BOYLE, G. POLITES & K. R. MAROTTI: Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 346: 74~76, 1990
5) ERIKSSON P.; B. KALLIN, F. M. VAN'T HOOFT, P. BÅVENHOLM, & A. HAMSTEN: Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc. Natl. Acad. Sci. USA 92: 1851~1855, 1995
6) WIMAN, B.: Plasminogen activator inhibitor 1 (PAI-1) in plasma: its role in thrombotic disease. Thromb. Haemost. 74: 71~76, 1995
7) HAMBERG, M.; W. H. GERWICK & P. A. ÅSEN: Linoleic acid metabolism in the red alga *Lithothamnion corallioides*: biosynthesis of 11(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid. Lipids 27: 487~493, 1992