Digestion of Endothelin-1 on Cultured Vascular Smooth Muscle Cells

Toshiyuki Edano, Koichi Arai, Tomoyuki Koshi, Takahiro Torii, Takeshi Ohshima, Mitsuteru Hirata, Masao Ohkuchi, and Tetsuro Okabe

Tokyo Research Laboratories, Kowa Co., Ltd., Noguchi-cho, Higashimurayama, Tokyo 189, Japan and The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo 7–3–1, Bunkyo-ku, Tokyo 113, Japan. Received July 23, 1993; accepted October 18, 1993

The degradation of $^{125}$I-endothelin-1 ( $^{125}$I-ET-1) was examined on cultured porcine aortic endothelial cell (EC) and rat vascular smooth muscle cell (SMC) by HPLC analysis. The degradation of ET-1 was observed on SMC and was slightly observed on EC. Membrane fractions of SMC had a strong potency for the degradation of ET-1 and retained activity even in plasma. This activity was inhibited with some of the noble inhibitors which were known as enkephalinase inhibitors. These results suggest that endothelin degradation enzyme on SMC is related to enkephalinase and that this enzyme plays a significant role in the degradation of ET-1 in vivo.

Keywords: endothelin-1 degradation; endothelin-1 digestion; vascular smooth muscle cell; cultured vascular smooth muscle cell; enkephalinase; neutral endopeptidase

Endothelin (ET)-1 which was isolated from cultured porcine endothelial cells (EC), has a potent and long-lasting vasoconstrictor activity on several species of blood vessels. Since the initial identification of ET-1, two other related peptides have been reported and designated ET-2 and ET-3, differing by 2 and 6 amino acid residues, respectively. All three peptides appear to be distinct gene products. Related peptides, vasoactive intestinal contractor (VIC) and Sarafotoxins (SRTX)'s isolated from intestine of mouse and the venom of snake respectively also belong to the ET family. ET seems to be related to several diseases such as essential hypertension, acute myocardial infarction, diabetes mellitus and uremia. It is also though to affect organs and tissues when produced locally in several tissues such as the aortic intima, lung and kidney inner medulla. Therefore, we considered that rapid inactivation of locally produced ET must be important in inhibiting the ET-induced disorders. Sirviö et al. reported that circulating ET-1 is rapidly cleared, primarily by the lung. However, the role of the lung in this clearance is controversial and there is little evidence for a clearance receptor at present. There have, on the other hand, been several reports about the degradation enzyme of ET: enkephalinase-like protease on smooth muscle cells (SMC), diamidase from human platelet and enkephalinase from rat kidney. Neutral endopeptidase on SMC membrane which actively metabolizes ET-1 was investigated using ET-1 binding assay. Digestion of ET family by enkephalinase which was purified from bovine or rat was analyzed by HPLC and the cleavage of ET by enkephalinase reportedly occurred at Ser$^2$–Leu$^3$, His$^6$–Leu$^{17}$ and Asp$^{18}$–Ile$^{19}$. Since no detailed reports have been published to date on a physiological study of ET degradation enzyme, we have examined kinetically the digestion of ET which was analyzed by HPLC using vascular cells, cultured EC and SMC, in vitro. The effect of several kinds of protease inhibitors was also determined.

MATERIALS AND METHODS

Materials were obtained from the following commercial sources and used according to the procedures given by the respective suppliers: (3-$^{125}$I)iodotyrosyl) $^{125}$I-ET-1, namely $^{125}$I-ET-1 (70 Tbid mmol) from Amersham (Tokyo, Japan); rat fetal aortic SMC (A10, ATCC®CRL 1476) from American Type Culture Collection; porcine EC were isolated as described; phosphoramidon, E-64, antipain and pepstatin A from Peptide Institute Inc. (Osaka, Japan); thorphan, captopril and prouroycin from Sigma Chemical Co. (St. Louis, U.S.A.).

Cell Culture SMC or EC was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (J R Scientific), 100 U/ml penicillin G and 100 µg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). The cells were cultured in 75 cm$^2$ flasks at 37°C under a humidified 95% air/5% CO$_2$ environment. Cells were subcultured at confluence with 0.25% trypsin, 0.02% EDTA in phosphate buffered saline (PBS), pH 7.4.

$^{125}$I-ET-1 Digestion Assay by Cultured Cells Following the attainment of confluence, the cells were washed with PBS and the medium was changed to 15 ml of fresh medium without FCS. $^{125}$I-ET-1 (approximately 1700 Bq) was added to the medium to initiate the hydrolysis reaction. Cells were incubated at 37°C for a specified period (see Results), and the medium was removed and centrifuged at 1000 x g for 10 min. The supernatant was acidified with 50% TFA to pH 2, and was applied on an Amprep C$^8$ column (500 mg). After washing with 0.1% TFA, the digestion mixture was eluted with 80% acetonitrile containing 0.1% TFA. The eluate was evaporated in vacuo, resolved in 100 µl of 0.1% TFA solution and analyzed by HPLC.

The hydrolysis of $^{125}$I-ET-1 in medium conditioned with SMC was determined as follows: After the cultures reached confluence, the cells were washed with PBS and incubated for 1 h in fresh medium without FCS. The conditioned medium was centrifuged at 1000 x g for 10 min. $^{125}$I-ET-1
was added to the supernatant. After 1 h incubation at 37°C, the reaction mixture was acidified, concentrated with Amprep C8 column and analyzed by HPLC as described above.

The Hydrolysis of $^{125}$I-ET-1 by Fractionated SMC
The SMC cells ($5 \times 10^6$ cells) were washed with PBS and scraped with a rubber policeman. After centrifugation at $1000 \times g$ for 10 min, the cells were suspended in 500 µl of PBS containing 0.25 M sucrose and sonicated for 30 s on ice. The cell lysate was centrifuged at 100000 $\times g$ for 1 h at 4°C. The precipitate as membrane fraction was washed twice with the above buffer and with 0.2 M KCl-4 mM EDTA, and resuspended in 100 µl of PBS. $^{125}$I-ET-1 was added to 50 µl of membrane fraction in 450 µl of 0.1 M PBS containing 0.5% Triton X-100 and 5% bovine serum albumin to prevent adsorption (buffer A), and incubated at 37°C for a specified period (see Results). The reaction was terminated by the addition of 3 µl of 50% TFA. The mixture was centrifuged at 5000 $\times g$ for 5 min and the supernatant was directly analyzed by HPLC.

To determine their effect, each of inhibitor was added individually to buffer A. Enzyme inhibitors used were phosphoramidon, thiorphan, EDTA, E-64, captopril, puromycin, antipain, chymostatin and pepstatin A.

Analysis by Reverse-phase High-Pressure Liquid Chromatography (HPLC) Sample was applied on a µBondosphere C18 column (3.9 mm x 15 cm) and eluted with a linear gradient of aq. acetonitrile (0 to 80%) in 0.1% TFA for 60 min at a flow rate of 0.4 ml/min. One milliliter fractions were collected and the radioactivity of each fraction was counted by an autowell gamma counter (ARC-251, Aloka). The retention time of native $^{125}$I-ET-1 was 42 min. Percent of residual $^{125}$I-ET-1 was calculated from the ratio of radioactivity of remaining $^{125}$I-ET-1 to the total eluate.

RESULTS AND DISCUSSION

The degradation of $^{125}$I-ET-1 was examined using serum-free cultures of vascular EC and SMC and the digestion curve is shown in Fig. 1A. The ET degradation activity on the SMC culture was investigated in short-term culture that seemed to be important physiologically (Fig. 1B). Fifty percent degradation of the added $^{125}$I-ET-1 in the SMC or EC culture occurred at 1 and 30 h, respectively. The degradation in vascular EC was not significant and was considered the effect of proteases of lysosome from dead cells. It is interesting that ET degradation occurred on SMC which was one of the target cells of ET, but did not occur in the short-term on EC which produced ET. To examine bounded or uptake $^{125}$I-ET-1 in SMC, the radioactivity of cells solubilized in 1 N NaOH was measured, and approximately 20% of the added radioactivity was recovered. Moreover, to see whether degradation of the added ET-1 was attributable to soluble factors released from the cultured cells or due to the cells themselves, the digestion activity in the culture medium conditioned with SMC was examined. Approximately 90% of the added $^{125}$I-ET-1 was recovered after incubation with the SMC conditioned medium. These results suggest that no significant activity is released into the culture medium from SMC. Thus, the digestive activity found in the SMC culture (Fig. 1) appears to be attributable to the intact cells themselves. These notions lead us to examine whether the ET degradation enzyme might localize in the membrane of SMC.

To characterize the digestive activity on the intact SMC, the membrane fraction of SMC was examined for ET degradation activity. The activity was found and not lost after washing the membrane fraction with KCl-EDTA solution to eliminate the possible contaminating cytoplasmic proteases (Fig. 2). These data suggest that the ET degradation enzyme is present in the membrane of SMC.
Fig. 3. Reverse-Phase HPLC Profiles of Products of 125I-ET-1 Degraded with Membrane Fraction

Four degraded products were recognized at a retention time of 27, 30, 34 and 36 min during incubation of 125I-ET-1 with the SMC membrane fraction (Fig. 3). The product at a retention time of 27 min increased significantly during the incubation, and was identified as 125I-Tyr by HPLC analysis (data not shown).

Interestingly, the ET degradation activity in the membrane fraction of SMC was not lost after incubation with blood plasma, suggesting that on SMC it can exert its function even in vivo.

Characterization of the ET degradation activity of SMC membrane fraction was carried out using several enzyme inhibitors. Table I shows the effects of proteinase inhibitors on the digestion of 125I-ET-1 with SMC membrane fraction. The activity was inhibited with 1 μM of thiorphan, phosphoramidon, and 1 mM of EDTA, respectively. The other inhibitors, captopril, antipain, and pepstatin A did not affect the degradation activity even at 100 μM. The ET degradation enzyme thus appears to be related to enkephalinase.

These observations suggest that ET-1 produced by the vascular EC is destroyed physiologically by the ET degradation enzyme on SMC, which is related to enkephalinase.

### REFERENCES