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Short Communication

Determination of endothelin-converting enzyme activity by high-performance liquid chromatography–on-line radioactive flow monitoring

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ABSTRACT

A rapid method to investigate the metabolism of ^{125}I-labelled or non-labelled human big endothelin to endothelin-1 by reversed-phase high-performance liquid chromatography (HPLC) and on-line radioactive flow monitoring and/or ultraviolet detection was developed. Samples were processed by solid-phase extraction (average recovery 70–80% for non-labelled and 20–25% for ^{125}I-labelled big endothelin and endothelin-1) followed by HPLC analysis (total analysis time 20 min). The method was successfully employed to monitor the conversion of big endothelin to endothelin-1 by various blood-borne cells, such as human polymorphonuclear leukocytes or monocytes/macrophages.

INTRODUCTION

Endothelin-1 (ET-1), a 21-amino acid peptide first isolated from cultered endothelial cells [1], is the most potent vasoconstrictor known and, thus, may be of considerable clinical importance (*cf.* ref. 2). ET-1 is formed via cleavage of its precursor big endothelin (bET), a 38-amino acid (human bET) or 39-amino acid peptide (porcine bET), by a protease or a group of proteases referred to as "endothelin-converting enzyme" (ECE). ECE activity has so far been ascribed to a variety of proteolytic enzymes including acidic proteases, such as pepsin [3], cathepsin-D [4] or cathepsin-E [5], neutral metalloproteases [6] as well as chymotrypsin-like proteases [7]. The pharmacological potency of ET-1 *in vitro* is usually two to three orders of magnitude greater than that of bET (*e.g.* ref. 8). When injected intravenously into anaesthetised rats, however, ET-1 and bET are almost equipotent vasopressor agents, implicating a rapid and substantial conversion of bET to ET-1 *in vivo* [9]. It is not clear, however, which class of proteolytic enzymes is responsible for this conversion and where the ECE activity is localised. A pre-requisite to address these questions experimentally is a rapid and sensitive assay for the simultaneous detection of bET and ET-1 as well as their metabolites. We have, therefore, developed a reversed-phase high-performance liquid chromatography (HPLC) technique which is described as follows.

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EXPERIMENTAL

Materials

Synthetic human bET (referred to as bET), porcine bET, human bET_{22-38} and ET-1 were obtained from the Peptide Institute (Osaka, Japan). Tyr^{13}-[^{125}I]bET and Tyr^{13}-[^{125}I]ET-1 (specific activity 2000 Ci/mmol) were purchased from Amersham International (Amersham, UK). The purity ($\geq 98\%$) of the non-labelled and ^{125}I-labelled endothelins was checked by HPLC analysis (see below). Pepstatin A was from Sigma (Poole, UK). All other chemicals and solvents were obtained in the highest commercially available quality from either Sigma or Merck (Dagenham, UK).

Cell incubations

Human polymorphonuclear leukocytes (PMNs) and mononuclear cells were prepared from citrated blood as previously described for rabbit PMNs [10]. The cells ($4 \cdot 10^6$ per ml; viability $>97\%$) were incubated in phosphate-buffered saline (pH 7.4) containing glucose (5.6 m*M*), $CaCl_2$ (1 m*M*), $MgCl_2$ (0.5 m*M*) and bET (10 μM final concentration) or [^{125}I]bET (0.4 n*M* final concentration) in a total volume of 250 μl for 0–120 min at 37°C. Incubations were terminated by acidification to pH 3.0 with trifluoroacetic acid (TFA) and addition of EDTA (5 m*M* final concentration) and pepstatin A (100 μM) to remove any residual Ca^{2+} and to prevent the activation of acidic proteases. After 10 min at 0–4°C, the samples were centrifuged at 10 000 *g* for 10 min followed by solid-phase extraction of the supernatant with octadecylsilica cartridges (Tech Elut from HPLC Technology, Macclesfield, UK). The extraction solvent (80% acetonitrile in 0.05% TFA–water) was evaporated under a stream of nitrogen and the residues were dissolved in 100 μl of 25% acetonitrile in 0.05% TFA–water.

Reversed-phase HPLC analysis

The samples were applied to a 250 mm × 4.6 mm I.D. Tosoh ODS-120T (TSK gel) HPLC column (from Anachem, Luton, UK) fitted with a 50 mm × 4.6 mm I.D. SynChropak RP-P guard-column (SynChrom from FSA Laboratory Supplies, Loughborough, UK). The column was isocratically eluted at 1.0 ml/min with acetonitrile–TFA–water (35:0.05:65 v/v/v) for 20 min followed by 100% methanol for 10 min. Radioactivity in the eluate was monitored with a Berthold LB 506 AT on-line radioactivity detector. Alternatively, the column was eluted at 1.0 ml/min with a binary mixed-linear gradient of 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) as follows: 0 min, 75% A–25% B; 2.5 min, 70% A–30% B; 12.5 min, 60% A–40% B; 15 min, 50% A–50% B; 20 min, 50% A–50% B; 20.1 min, 100% methanol; 29.9 min, 100% methanol; 30 min, 75% A–25% B. In addition to the radioactive on-line flow monitoring, the UV absorbance of the eluate was continuously monitored at 214 nm by using a Waters Model 441 UV detector.

RESULTS AND DISCUSSION

The recovery of authentic bET, ET-1, [^{125}I]bET and [^{125}I]ET-1 following solid-phase extraction, reconstitution in 25% acetonitrile and application to the HPLC column was (mean ± S.D.) 85 ± 3% ($n=3$), 67 ± 5% ($n=3$), 21 ± 3% ($n=10$) and 25 ± 6% ($n=8$), respectively. The relatively poor recovery of the ^{125}I-labelled peptides may have been caused by substantial losses to plastic or glassware during the sample processing. Elution of the octadecylsilica cartridges with 0.05% TFA in methanol did not improve recovery, but resulted in an up to 80% conversion of both bET and ET-1 to a chromatographically distinct compound, possibly the corresponding methyl ester (data not shown).

Under isocratic conditions, bET, ET-1, [^{125}I]bET and [^{125}I]ET-1 were very well resolved, eluting at (mean ± S.D.) 6.3 ± 1.0 min ($n=3$), 11.5 ± 2.0 min ($n=4$), 8.1 ± 1.4 min ($n=3$) and 17.4 ± 2.4 min ($n=4$), respectively. However, the relatively high coefficient of variation, probably due to small differences in the composition of the solvent, and the fact that bET_{22-38} eluted with the solvent front, prompted us to employ a mixed-linear gradient instead. Under these conditions (Fig. 1), non-labelled bET_{22-38}, human or porcine bET and ET-1 eluted at 4.5 ± 0.3 min ($n=11$), 13.8 ± 0.3 min ($n=11$) and 16.0 ± 0.3

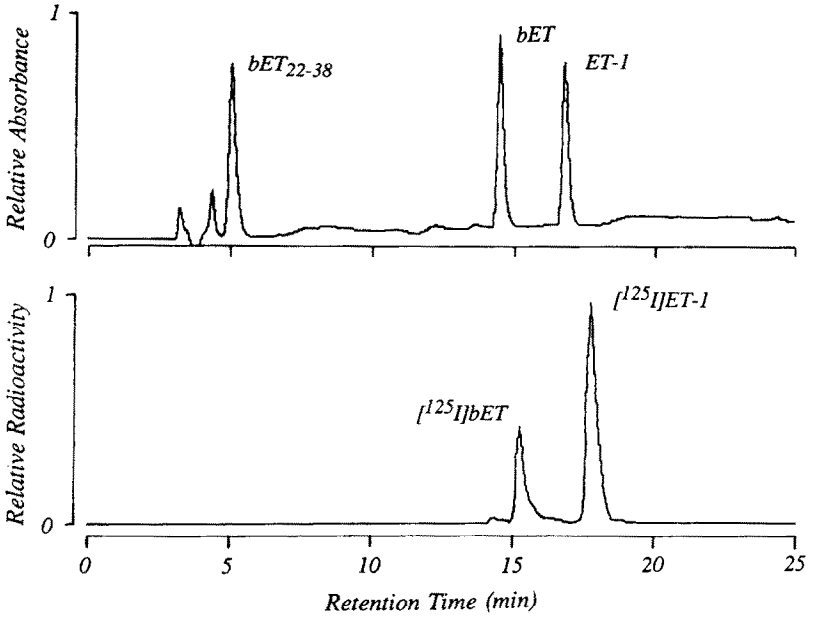

Fig. 1. Reversed-phase HPLC analysis (gradient elution) of synthetic non-labelled (UV detection; upper panel) and ^{125}I-labelled (on-line radioactive flow monitoring; lower panel) endothelin standards. The following amounts were injected: bET_{22-38}, 1 nmol; bET, 0.5 nmol; ET-1, 1 nmol; [^{125}I]bET, 25 nCi corresponding to 25 fmol; and [^{125}I]ET-1, 50 nCi corresponding to 50 fmol.

min ($n = 11$), respectively, whereas the retention times of [^{125}I]bET (15.1 ± 0.4 min; $n = 15$) and [^{125}I]ET-1 (17.5 ± 0.4 min; $n = 15$) were again longer.

Standard calibrations curves for bET, ET-1 and bET_{22-38} were linear over a range of 0.01 to 10 nmol. The detection limit was 5 pmol for bET, 10 pmol for ET-1 and bET_{22-38} (UV detection) and 1 fmol for [^{125}I]bET and [^{125}I]ET-1 (radioactive on-line flow monitoring), respectively. Electrochemical detection (oxidative mode, 1050 mV, EG&G Model 400 detector) instead of UV detection did not improve the detection limit for bET (5 pmol), ET-1 or bET_{22-38} (10 pmol). Theoretically, the HPLC method also permits the detection of Tyr^{31}-[^{125}I]bET_{22-38}. However, neither Tyr^{31}-[^{125}I]bET_{22-38} nor Tyr^{13},Tyr^{31}-[^{125}I]bET are as yet commercially available, so that Tyr^{31}-[^{125}I]bET (Amersham International) would have to be used instead, and this will preclude the detection of the non-labelled ET-1 fragment.

The resolution and speed of the newly developed HPLC method for the separation of bET and ET-1 appears to be significantly better than those described previously. Most of these methods use wide-pore octadecylsilica columns, usually with a pore size of 300 Å. We have tested similar columns and found that, at the same speed of elution, their resolution was unsatisfactory when compared to the 120 Å column described herein. Presumably the smaller pore size and, hence, the different size-exclusion effect of the 120 Å column facilitates the elution of bET as a symmetric peak before ET-1, thereby providing the basis for the rapid separation of these two peptides.

As depicted in Figs. 2 and 3, the HPLC method was successfully used to monitor the conversion of bET to ET-1 as well as the degradation of both of these peptides by various blood-borne human cells, such as PMNs and mononuclear cells. Moreover, the HPLC fractions corresponding to bET and ET-1 were collected, lyophylized and subjected to electrospray–mass spectrometry analysis, which confirmed their molecular weight as 4283 and 2492, respectively. A portion of the lyophylisate was also tested for vasoconstrictor activity in a superfusion bioassay system consisting of two rabbit jugular veins arranged in a cascade [8], and demonstrated a potent vasoconstriction with the HPLC fraction corresponding

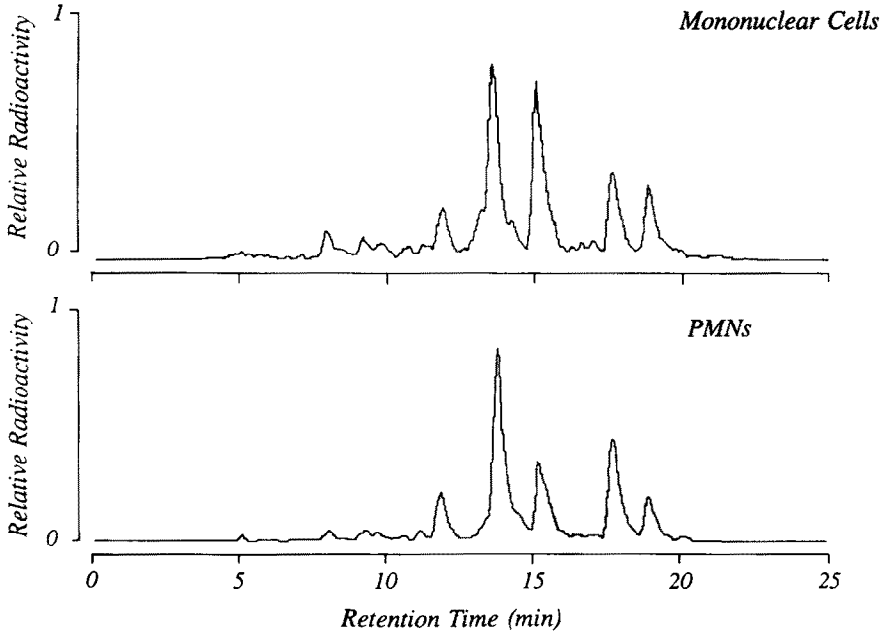

Fig. 2. Reversed-phase HPLC–on-line radioactive flow monitoring analysis of the metabolism of [^{125}I]bET by non-activated human PMNs (lower panel) or mononuclear cells (upper panel). For experimental details refer to Experimental. The figure depicts two typical chromatograms (gradient elution) of a 30-min incubation of either cell type with 0.4 n*M* [^{125}I]bET from at least three individual experiments with cells from different donors. Note [^{125}I]bET and [^{125}I]ET-1 elute at 15.1 and 17.6 min, respectively.

to ET-1, but not bET (data not shown). Interestingly, both leukocyte cell types not only produced ET-1, but also degraded both bET and ET-1 to a similar extent, resulting in an almost identical peptide fragmentation pattern (Fig. 2).

CONCLUSION

We have developed a rapid (total analysis time 20 min) and sensitive (1 fmol detection limit when using ^{125}I-labelled endothelins) HPLC method to study the conversion of bET to ET-1 as well as the degradation of these two peptides by various cells, tissues and subcellular fractions *in vitro*. The same methodology is equally well suited to monitor the fate of [^{125}I]bET *in vivo* or to analyse its metabolism by isolated organs *in vitro* (data not shown). Moreover, by combining radioactive on-line flow monitoring with UV detection, the concentration of bET or ET-1 can be varied over

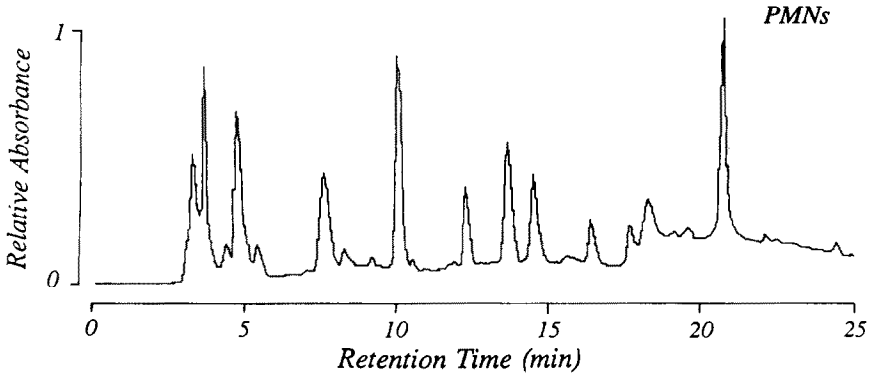

Fig. 3. Reversed-phase HPLC–UV detection analysis of the metabolism of bET by non-activated human PMNs. For experimental details refer to Experimental. The figure depicts a typical chromatogram (gradient elution) of a 60-min incubation of isolated human PMNs with 10 μM bET from at least three individual experiments with cells from different donors. Note bET_{22-38}, bET and ET-1 elute at 4.8, 14.3 and 16.4 min, respectively.

several orders of magnitude, *i.e.* from 0.1 n*M* to 1 m*M*. However, the distinct chromatographic properties of the ^{125}I-labelled and non-labelled peptides is also reflected by their metabolism, *i.e.* PMNs do not cleave [^{125}I]bET in the presence of an excess of bET (data not shown), thus precluding the use of mixtures of labelled and non-labelled peptide(s). In addition, it should be stressed that although on-line radioactive flow monitoring is almost as sensitive as radioimmunoassay, UV detection or electrochemical detection of peptides of this size in principle does not permit the determination in the low picomolar range of endogenous levels of bET or ET-1 in cells or tissues or plasma [2]. The main advantage of the present method, however, is that it allows a quantitative analysis of the rather complex proteolytic fragmentation of bET which besides ET-1 may produce other biologically active peptides.

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REFERENCES

1 M. Yanagiswa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazake, K. Goto and T. Masaki, *Nature (London)*, 332 (1988) 411.
2 D. J. Stewart, R. D. Levy, P. Cernacek and D. Langleben, *Ann. Intern. Med.*, 114 (1991) 464.
3 M. Takaoka, Y. Takenobu, Y. Miyata, R. Ikegawa, Y. Matsumura and S. Morimoto, *Biochem. Biophys. Res. Commun.*, 166 (1990) 436.
4 Y. Matsumura, R. Ikegawa, M. Takakoa and S. Morimoto, *Biochem. Biophys. Res. Commun.*, 167 (1990) 203.
5 W. E. Lees, S. Kalinka, J. Meech, S. J. Capper, N. D. Cook and J. Kray, *FEBS Lett.*, 273 (1990) 99.
6 K. Okada, Y. Miyazaki, J. Takada, K. Matsuyama, T. Yamaki and M. Yano, *Biochem. Biophys. Res. Commun*, 171 (1990) 1192.
7 M. Yanagisawa and T. Masaki, *Biochem. Pharmacol.*, 38 (1989) 1877.
8 W. C. Sessa, S. Kaw, M. Hecker and J. R. Vane, *Biochem. Biophys. Res. Commun.*, 174 (1991) 613.
9 Y. Matsumura, K. Hisaki, M. Takakoa and S. Morimoto, *Eur. J. Pharmacol.*, 185 (1990) 103.
10 W. C. Sessa and K. M. Mullane, *Br. J. Pharmacol.*, 99 (1990) 553.