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Presence of immunoreactive endothelin in human milk

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Endothelin-like immunoreactivity was detected in human milk at a concentration of 6.8 ± 1.6 pmol/l (mean ± SEM; $n = 16$) using a highly sensitive radioimmunoassay. Gel filtration and fast protein liquid chromatography (FPLC) verified the identity of the endothelin. FPLC revealed 4 peaks, one eluting just after the void volume, and the other three in the positions of endothelin-1, -2, and -3, respectively.

Endothelin; Chromatography; Human milk; Radioimmunoassay

1. INTRODUCTION

Endothelin-1 (ET-1), a 21 amino acid peptide, is a potent and long-lasting vasoconstrictor. It was originally isolated, and its structure determined, from the conditioned medium of cultured porcine endothelial cells [1]. Subsequently, a family of ET-like peptides, namely ET-1, ET-2 and ET-3, which have similar but not identical structures, were found to be encoded by three distinct genes, each present in the human, rat and porcine genome [2]. ET-like immunoreactivity has been detected in human plasma [3] and urine [4]. Many peptides and growth factors are present in mammalian milk [5], and physiological roles have been suggested for them in the lactating mother and the suckling neonate [6–9]. Using our recently developed, sensitive and specific radioimmunoassay [10] together with gel chromatography and fast protein liquid chromatography (FPLC), we have investigated the concentration and molecular forms of immunoreactive endothelin (IR-ET) in human milk.

2. MATERIALS AND METHODS

2.1. *Milk extraction*

Fresh human milk samples were obtained from 16 lactating mothers after the colostrum phase was completed and stored at −20°C immediately. All subjects were less than eight days postpartum. After thawing, milk samples were centrifuged at 4°C for 20 min to separate the fat and aqueous layers. The aqueous milk (5 ml) was acidified with 2 ml 4% acetic acid and extracted immediately using Sep-Pak C18 cartridges (Waters Associates, MA, USA) as previously described [10]. For chromatographic characterization of milk IR-ET, a 100-ml aliquot of pooled human milk was extracted as above, and applied to gel permeation and FPLC, respectively. The recovery of synthetic ET added to human milk was 62.4 ± 4.4% ($n = 5$) following the extraction procedure.

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2.2. *Gel permeation chromatography*

Reconstituted Sep-Paked human milk extracts were centrifuged to remove particulate material and loaded onto a Sephadex G-25 Superfine column (0.9 × 60 cm, Pharmacia, Uppsala, Sweden). The column was eluted at a flow rate of 3.5 ml/h at 4°C in 60 mM phosphate buffer (pH 7.4) containing 10 mM EDTA, 7 mM sodium azide, 0.2 M NaCl and 0.3% (w/v) bovine serum albumin. The positions of void volume and total volume were determined by dextran blue and $Na^{125}I$, respectively. 0.7-ml fractions were collected for RIA. The recovery of IR-ET in human milk extracted from the column was 80%.

2.3. *Fast protein liquid chromatography (FPLC)*

Human milk extracts were reconstituted in 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and centrifuged prior to analysis by FPLC with a high-resolution reverse-phase (Pep RPC HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden). The column was eluted with a gradient of acetonitrile from 15% to 35% (v/v) in water with 0.1% TFA over 1 h at a flow rate of 1 ml/min per fraction. Samples of each fraction were dried in a Savant vacuum centrifuge, reconstituted in assay buffer, and assayed. The recovery of IR-ET in human milk extract from the column was 95%.

2.4. *Stability of ET-1, ET-2, and ET-3 in human milk*

To examine the stability of ET in human milk, ET-1, 2, and 3 were incubated (1 pmol/ml milk) in human milk at room temperature for 5 h. Then the milk samples were extracted by Sep-Pak C18 cartridges and loaded onto the FPLC. The IR-ET in each fraction was measured by RIA.

2.5. *Radioimmunoassay of IR-ET*

Radioimmunoassay of ET has been described previously in detail [10], but the antibody used in the present study was obtained from a subsequent bleed of the immunogenic rabbit and gave increased sensitivity. This assay could detect changes of 0.1 fmol/tube at 95% confidence with duplicate tubes. The antibody showed 60% cross-reactivity with ET-2 and 70% with ET-3 (Peptide Institute, Osaka, Japan) but no cross-reaction with α-human atrial natriuretic peptide, neuropeptide Y, substance P, vasoactive intestinal peptide, peptide histidine methionine, glucagon, angiotensin II and ACTH.

3. RESULTS AND DISCUSSION

The dilution curves of human milk extract and the void volume peak from FPLC of human milk extract

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paralleled the standard curve (fig.1). The mean concentration of IR-ET in the milk from lactating mothers was 6.8 ± 1.6 pmol/l (range, 0.5–21.2 pmol/l, $n = 16$). This is higher than the concentration found in normal human plasma (0.54 ± 0.05 pmol/l, $n = 19$) [11]. The presence of IR-ET in human milk raises not only the question of its role there but also of its origin. The higher concentration of IR-ET in milk as compared to plasma implies either an active concentrating mechanism in the mammary gland or an extravascular origin for this peptide.

Gel permeation chromatography of pooled human milk extract showed two immunoreactive components (fig.2). The first minor component which eluted just after the void volume could be a large molecular weight form of IR-ET and may be a precursor of ET. The second major component comprised most of the eluted immunoreactivity in a broad peak sparing the elution positions of ET-1, ET-2 and ET-3. Sephadex G-25 gives different elution positions for ET-1, ET-2 and ET-3 in spite of their similar molecular weights, presumably due to their ring structure.

FPLC of milk extracts resulted in 4 peaks (fig.3A), one just after the void volume and the other three in the positions of ET-1, ET-2 and ET-3, respectively. The nature of the void volume peak is unknown. However, we have found a similar peak of FPLC from human plasma [11] and from human urine extracts (unpublished observations). Incubation of exogenous ET-1, ET-2 and ET-3 in human milk for 5 h did not produce any material eluting in the void volume peak position, so it is unlikely to be an extraction artifact or a degraded form of ET. As the 2-fold serial dilution of void volume peak material paralleled the standard curve (fig.1), we suggest that this peak represents a precursor of ET.

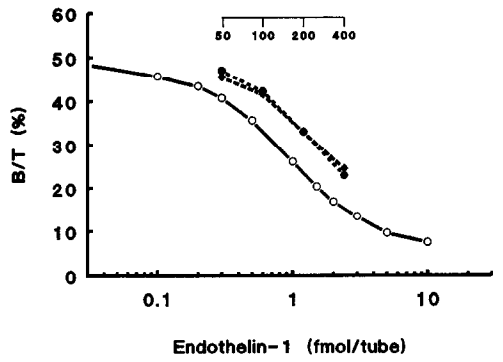

Fig.1. A standard curve for ET (○—○) and 2-fold serial dilution curves of normal human milk extract (●—●) and the second fraction of human milk extract from FPLC (◆—◆). Serial dilutions of the original milk volume are denoted above the curves.

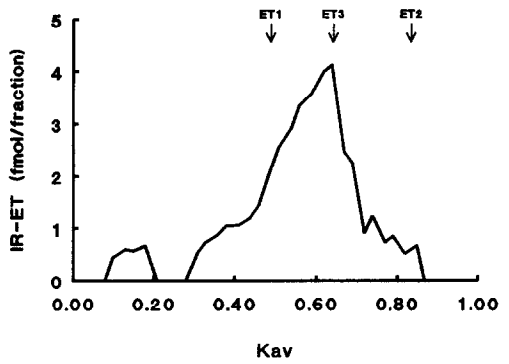

Fig.2. Sephadex G-25 column chromatography of IR-ET in a pooled normal human milk extract. ET-1, ET-2, and ET-3, the elution positions of standard ET-1, 2 and 3, respectively.

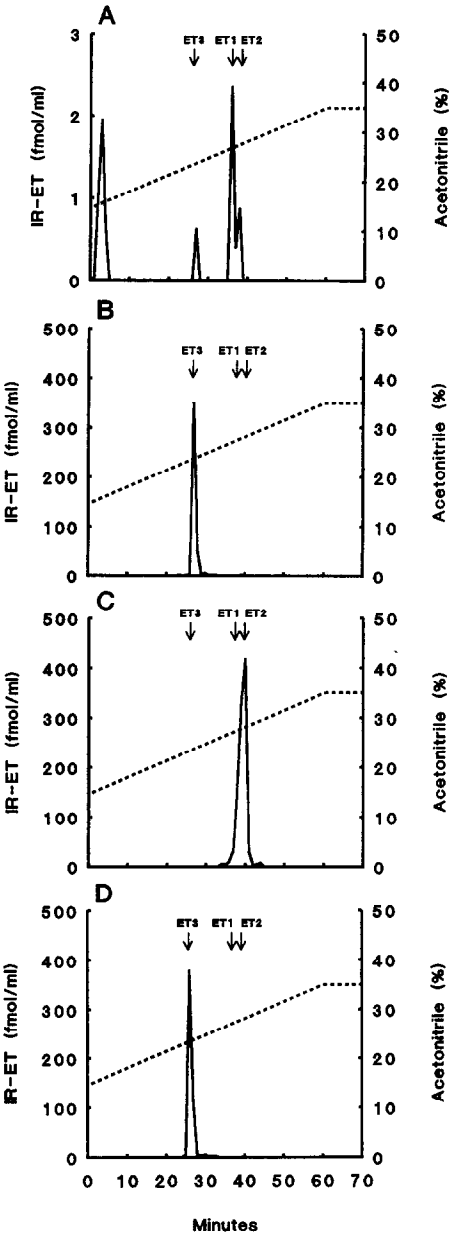

Fig.3. FPLC of IR-ET in: (A) a pooled normal human milk extract; (B) ET-1 incubated in human milk at room temperature for 5 h; (C) ET-2 incubated in human milk for 5 h; (D) ET-3 incubated in human milk for 5 h. The dotted lines show a linear gradient of acetonitrile from 15% to 35%.

The total content of IR-ET in human milk with exogenous ET-1 decreased to 70% of its original value after 5 h incubation at room temperature (data not shown). FPLC showed that ET-1 was converted to a form which eluted in a single peak at the position of ET-3 (fig.3B). This suggests that ET-1 had become oxidized. In contrast, the concentration of exogenous ET-2 and ET-3 did not decrease after 5 h incubation in milk (data not shown) and the elution profiles of FPLC showed a single peak in the positions of ET-2 (fig.3C) and ET-3, respectively (fig.3D). From these results, we cannot be certain that ET-3 exists in human milk, as a peak corresponding to ET-3 is produced by ET-1 over a period of time. In addition, as the antiserum to ET-1 used in this study showed 70% cross-reactivity with ET-3 and 60% with ET-2, greater amounts of IR-ET may be present in milk than we have been able to measure.

ET has been reported to cause contraction in the rat gut [12] and we have recently shown that ET receptors are present in the gastrointestinal tract of rats [13]. It is therefore possible that ET in milk may have a physiological role in the development and motility of the gastrointestinal tract of the suckling neonate.

In conclusion, the present study demonstrates for the first time the presence of IR-ET in human milk. Further studies are needed to investigate its possible functions in the lactating mother and the neonate.

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