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Rapid receptor-mediated catabolism of ^{125}I-atrial natriuretic factor by vascular endothelial cells

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The binding, internalization and degradation of 200 pM monoiodinated human atrial natriuretic factor-(99–126) (^{125}I-hANF) by cultured bovine aortic endothelial cells (BAECs) were studied at 37 °C. ^{125}I-hANF was rapidly cleared from the extracellular medium ($t_{\frac{1}{2}} \sim 10$ min), whereas preincubation of the cells in the presence of 20 mM-NH_4Cl or 0.2 mM-chloroquine resulted in a significant inhibition of this process. The BAECs rapidly produce three major degradation products of ^{125}I-hANF, namely [^{125}I]iodotyrosine^{126} (^{125}I-Y), Arg^{125}-[^{125}I]iodotyrosine^{126} (^{125}I-RY) and Phe^{124}-Arg^{125}-[^{125}I]iodotyrosine^{126}(^{125}I-FRY), which were detected in the extracellular medium. NH_4Cl and chloroquine acted to inhibit the generation of ^{125}I-Y and ^{125}I-RY, but not that of ^{125}I-FRY. Furthermore, excess unlabelled hANF (300 nM) completely blocked the rapid production of ^{125}I-Y and ^{125}I-RY in the first 5 min, but only partially (49 %) inhibited the generation of ^{125}I-FRY. Thus, in contrast with our previous findings with cultured smooth-muscle cells [Johnson, Arik & Foster (1989) J. Biol. Chem. **264**, 11637–11642], BAECs bind, internalize and rapidly degrade ^{125}I-hANF, resulting in the release of ^{125}I-Y and ^{125}I-RY into the extracellular medium. Similarly to smooth-muscle cells, the BAECs generate ^{125}I-FRY from ^{125}I-hANF via an extracellular proteolytic event. The rapidity of the receptor-mediated process and its sensitivity to NH_4Cl and chloroquine suggest that the ^{125}I-hANF is proteolytically processed in the endosomes of BAECs and that its receptors cycle between the cell surface and intracellular stores.

INTRODUCTION

Atrial natriuretic factor (ANF), initially discovered by deBold *et al.* (1981), is a peptide hormone that elicits multiple biological effects, which include diuresis, natriuresis, vasorelaxation and inhibition of aldosterone secretion (for reviews, see Ballerman & Brenner, 1985; Cantin & Genest, 1985; Inagami, 1989). ANF is produced in the atria (Kangawa *et al.* 1984; Thibault *et al.*, 1987) as a 126-residue precursor (pro-ANF) and is processed to the major plasma circulating form, ANF-(99–126) (Thibault *et al.*, 1985; Schwartz *et al.*, 1985). ANF is secreted by atrial cardiocytes into the bloodstream, where it travels to target tissues, such as kidney, adrenal gland and vascular smooth muscle, which contain specific high-affinity receptors (DeLéan *et al.* 1984; Napier *et al.*, 1984; Hori *et al.*, 1985). Many of the biological effects of ANF are believed to be mediated through the generation of intracellular cyclic GMP via the stimulation of particulate guanylate cyclase (Hamet *et al.*, 1984, 1986; Waldman *et al.*, 1984). Multiple studies suggest that not all ANF receptor sites are coupled to the generation of cyclic GMP (Leitman *et al.*, 1986; Scarborough *et al.*, 1986; Takayanagi *et al.*, 1987), and Maack *et al.* (1987) have proposed that this non-coupled site functions to clear and store ANF.

The use of ANF as a therapeutic agent in various pathological conditions is limited since administration *in vivo* results in a very rapid clearance of the peptide from the circulation (half-lives of seconds to minutes) (Tang *et al.*, 1984; Luft *et al.*, 1986; Murthy *et al.*, 1986*a*,*b*; Yandle *et al.*, 1986). Little is known about the processes that rapidly clear ANF from the bloodstream. Studies indicate that ^{125}I-ANF binds to its receptor and is endocytosed in cultured smooth-muscle cells (Hirata *et al.*, 1985; Napier *et al.*, 1986; Johnson *et al.*, 1989). Proteolysis of ANF by purified kidney membranes (Koehn *et al.*, 1987; Olins *et al.*, 1987), atrial tissue extracts (Harris & Wilson, 1984) and cultured smooth-muscle cells (Johnson *et al.*, 1989) has been observed. An ectoenzyme of the renal brush border, endopeptidase-24.11, appears to be involved in the degradation and inactivation of ANF (Stephenson & Kenny, 1987; Kenny & Stephenson, 1988; Sonnenberg *et al.*, 1988). Although the kidney is a likely site for some of the degradation of ANF, animals in which the kidneys have been removed are still able to clear exogenously administered ANF rapidly (Luft *et al.*, 1986; Murthy *et al.*, 1986*b*).

The goal of research in our laboratory is to understand better the metabolic fate of ANF in the vasculature using cell-culture models. We have previously studied the metabolism of radiolabelled ANF by cultured vascular smooth-muscle cells (Johnson *et al.*, 1989), and here we have studied the metabolism of radiolabelled ANF by cultured vascular endothelial cells so as to compare and contrast the ways these two types of cells process the radiolabelled hormone. Cultured bovine aortic endothelial cells (BAECs) are rich in high-affinity ANF receptor sites (Schenk *et al.*, 1985; Leitman & Murad, 1986; Leitman *et al.*, 1986), although little is known about the metabolism of ANF by these cells. In the work described here, monoiodinated human ANF-(99–126) (^{125}I-hANF) was added to the cells at a concentration of radiolabelled ANF near the physiological range (Ballerman & Brenner, 1985). Receptor-mediated cell-surface binding and receptor-mediated endocytosis of the ^{125}I-hANF were monitored as a function of time. Further, intact ^{125}I-hANF and its degradation products were quantified by analytical reverse-phase h.p.l.c. NH_4Cl, chloroquine and excess unlabelled hANF were used to detect degradative mechanisms that involve the receptor-mediated endocytosis, intracellular processing and breakdown of ^{125}I-hANF. The results described here indicate that ^{125}I-hANF binds to receptors on BAECs, is internalized and is rapidly

Abbreviations used: ANF, atrial natriuretic factor-(99–126); hANF, human atrial natriuretic factor-(99–126); ^{125}I-Y, [^{125}I]iodotyrosine^{126}; ^{125}I-RY, Arg^{125}-[^{125}I]iodotyrosine^{126}; ^{125}I-FRY, Phe^{124}-Arg^{125}-[^{125}I]iodotyrosine^{126}; BAECs, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium.

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degraded, resulting in the release of degradation products into the extracellular medium. Similarly to our findings with cultured smooth-muscle cells (Johnson *et al.*, 1989), the BAECs also produce a peptidase that removes the *C*-terminal tripeptide from ^{125}I-hANF.

EXPERIMENTAL

Materials

The materials used in the experiments were the same as those described by Johnson *et al.* (1989).

Preparation of iodinated standards

Arginyliodotyrosine and phenylalanylarginyliodotyrosine were prepared by the method of Johnson *et al.* (1989).

Cell culture

BAECs were prepared as described by Schwartz (1978) and grown at 37 °C in 10% (v/v) calf serum, 1% penicillin (5000 units/ml)/streptomycin (5000 μg/ml) solution (catalogue no. 600–5070), 1% 200 mM-L-glutamine (catalogue no. 320–5030) in RPMI 1640 (catalogue no. 320-1875PJ) (Gibco, Grand Island, NY, U.S.A.). Cell monolayers were grown to confluence (349 500 ± 36 800 cells/well; mean ± S.D., n = 10) in 12-well plates (22 mm-diameter well) in a CO_2/air (1:19) atmosphere. Cells in passage 8 were used for the experiments.

Surface binding and internalization of ^{125}I-hANF

Surface binding and internalization of ^{125}I-hANF were determined as described in Johnson *et al.* (1989). Cellular protein per well was quantified by the method of Lowry *et al.* (1951), with BSA as standard, to confirm that a constant number of cells had been plated into each well.

Analysis of medium by analytical reverse-phase h.p.l.c.

The acidified cell-free medium (100 μl) was analysed by using the h.p.l.c. system described in Johnson *et al.* (1989). A 0.39 cm × 30 cm μBondapak C_{18} column (Waters) was equilibrated with 0.1% trifluoroacetic acid/water and peptides were eluted at ambient temperature with a linear gradient of 15–35% (v/v) acetonitrile (with 0.1% trifluoroacetic acid) over 38 min.

RESULTS

NH_4Cl and chloroquine inhibit the rapid clearance of ^{125}I-hANF from the extracellular medium by cultured BAECs

The addition of 200 pM-^{125}I-hANF (200 fmol/well) to the cultured BAECs at 37 °C resulted in a rapid clearance of the intact radiolabelled hormone from the extracellular medium (Fig. 1). The half-life ($t_{\frac{1}{2}}$) of this process was approx. 10 min (Fig. 1, ●). Preincubation and addition of ^{125}I-hANF in the presence of 20 mM-NH_4Cl or 0.2 mM-chloroquine resulted in a significant inhibition of the clearance of ^{125}I-hANF from the extracellular medium by BAECs (Fig. 1). Only 46 and 48% of ^{125}I-hANF were cleared from the medium relative to control in the first 5 min for NH_4Cl and chloroquine respectively. In 20 min, approx. 117, 63 and 68 fmol of intact ^{125}I-hANF were cleared from the extracellular medium by control cells, cells preincubated in 20 mM-NH_4Cl and cells preincubated in 0.2 mM-chloroquine respectively.

^{125}I-Y, ^{125}I-RY and ^{125}I-FRY rapidly appear in the extracellular medium

Within 2 min after the addition of 200 pM-^{125}I-hANF to the cell monolayers at 37 °C, three predominant degradation pro-

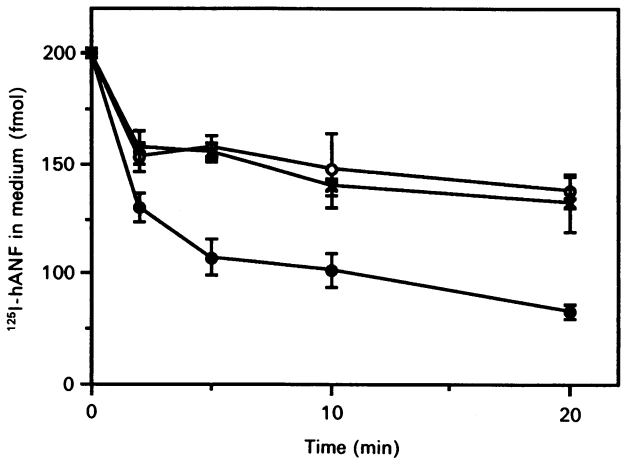

Fig. 1. Clearance of 200 pM-^{125}I-hANF from medium at 37 °C by cultured BAECs

Intact ^{125}I-hANF in the extracellular medium (per well) was quantified by reverse-phase h.p.l.c. as described in the Experimental section. Aliquots of the medium (100 μl) for control wells (●), wells preincubated in 20 mM-NH_4Cl (○) and wells preincubated in 0.2 mM-chloroquine (×) were analysed. Results are means ± S.D. of three determinations.

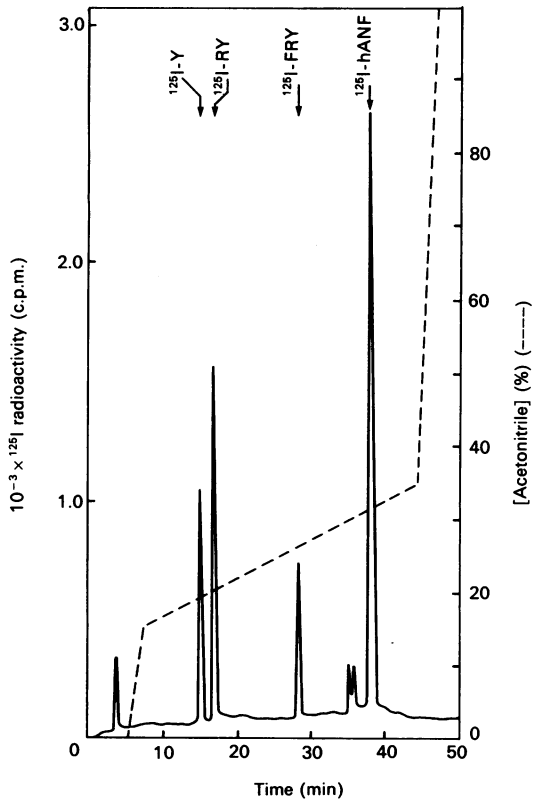

Fig. 2. Reverse-phase h.p.l.c. analysis of medium 20 min after addition of 200 pM-^{125}I-hANF to cultured BAECs at 37 °C

The chromatogram shows the analysis of a 100 μl aliquot of the medium as described in the Experimental section. Radioactivity was monitored as a function of time (continuous line). The acetonitrile gradient used to elute the peptides is represented by the broken line, and the identities of the known radiolabelled peptides are denoted at the top of the Figure by arrows. The peak that was eluted with the void volume of the h.p.l.c. system (at approx. 3.5 min) is radioactive iodide, a minor contaminant of the commercially purchased ^{125}I-hANF.

ducts containing the radiolabeled *C*-terminal Tyr^{126} residue appeared in the medium. These degradation products were easily resolved by analytical reverse-phase h.p.l.c., as seen in Fig. 2. These products were [^{125}I]iodotyrosine^{126} (^{125}I-Y), Arg^{125}-[^{125}I]iodotyrosine^{126} (^{125}I-RY) and Phe^{124}-Arg^{125}-[^{125}I]iodotyrosine^{126} (^{125}I-FRY). We have previously observed and identified these products being produced from ^{125}I-hANF by cultured smooth-muscle cells (Johnson *et al.*, 1989). The small amounts of radiolabelled degradation products generated in these endothelial-cell assays (< 100 fmol) did not permit a direct characterization of these products by amino acid analysis or Edman degradation. However, knowing the position of the radiolabel in the ^{125}I-hANF (*C*-terminal Tyr^{126}) did allow us to confirm the identities of the degradation products generated by the BAECs. Retention times on analytical reverse-phase h.p.l.c. for these degradation products were not altered after reduction by 10 mM-dithiothreitol, indicating that all cleavages had occurred *C*-terminal to the disulphide bond formed between Cys^{105} and Cys^{121}. The degradation products are co-eluted exactly with the synthetic standards iodotyrosine, arginyliodotyrosine and phenylalanylarginyliodotyrosine. Further, digestion of the putative ^{125}I-FRY generated by endothelial cells with aminopeptidase M generated ^{125}I-RY, followed by ^{125}I-Y, whereas digestion of the putative ^{125}I-RY only generated ^{125}I-Y. ^{125}I-Y was found to be insensitive to all exopeptidases and endopeptidases tested. These results clearly demonstrated that these products were derived from the *C*-terminal region of ^{125}I-hANF and represent the *C*-terminal amino acid (^{125}I-Y), dipeptide (^{125}I-RY) and tripeptide (^{125}I-FRY). It should also be noted that, in some h.p.l.c. analyses, we observed the production of two peaks with elution times of approx. 36 and 37 minutes (Fig. 2). These peaks never accumulated to a large extent and in many analyses were not produced at all.

NH_4Cl and chloroquine inhibit the generation of ^{125}I-Y and ^{125}I-RY, but not that of ^{125}I-FRY

Both ^{125}I-Y (Fig. 3, ●) and ^{125}I-RY (Fig. 4, ●) were released into the medium, and their quantity increased in a time-dependent fashion. These two degradation products were rapidly generated, and in 5 min had achieved 65 and 69% of the maximal value for ^{125}I-Y and ^{125}I-RY respectively. The appearance of these radiolabelled degradation products in the extracellular medium was found to be inhibited if the cells were preincubated in 20 mM-NH_4Cl or 0.2 mM-chloroquine for 30 min (Figs. 3 and 4). Furthermore, this inhibition was most dramatic in the first few minutes of the experiment. The *C*-terminal tripeptide, ^{125}I-FRY, was found to be rapidly produced in the medium and its quantity decreased slightly as a function of time (Fig. 5, ●). In contrast with ^{125}I-Y and ^{125}I-RY, the amount of ^{125}I-FRY detected in the medium was not decreased when the cells were preincubated in 20 mM-NH_4Cl or 0.2 mM-chloroquine (Fig. 5).

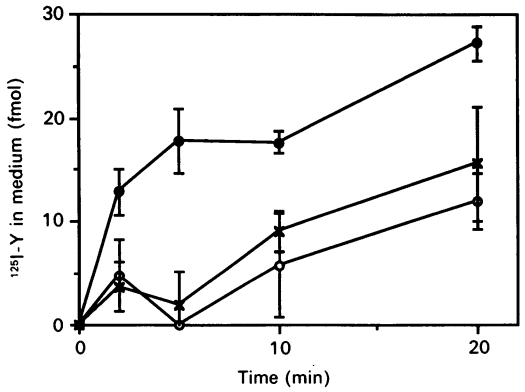

Fig. 3. Generation of ^{125}I-Y from ^{125}I-hANF by cultured BAECs

^{125}I-hANF (200 pM, 200 fmol/well) was added to the confluent cell monolayers at 37 °C, and the production of ^{125}I-Y/well was quantified by h.p.l.c. as described in the Experimental section. Aliquots (100 μl) of the medium from control wells (●), wells preincubated in 20 mM-NH_4Cl (○) and wells preincubated in 0.2 mM-chloroquine (×) were analysed. Results are means ± S.D. of three determinations.

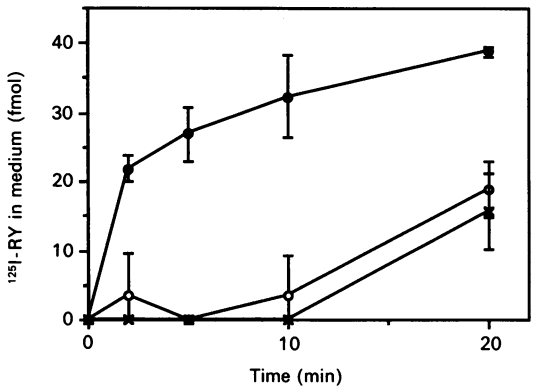

Fig. 4. Generation of ^{125}I-RY from ^{125}I-hANF by cultured BAECs

^{125}I-hANF (200 pM, 200 fmol/well) was added to the confluent cell monolayers at 37 °C and the production of ^{125}I-RY per well was quantified by h.p.l.c. as described in the Experimental section. Other details are as for Fig. 3.

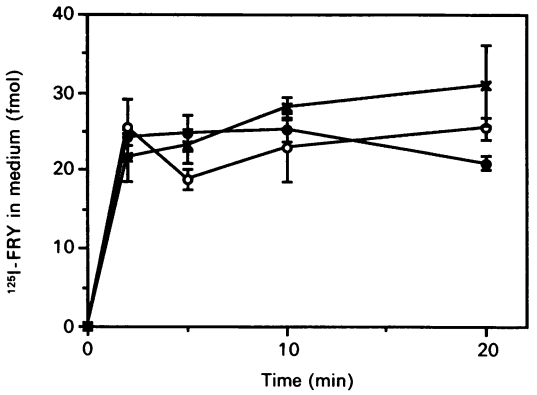

Fig. 5. Generation of ^{125}I-FRY from ^{125}I-hANF by cultured BAECs

^{125}I-hANF (200 pM, 200 fmol/well) was added to the confluent cell monolayers at 37 °C and the production of ^{125}I-FRY per well was quantified by h.p.l.c. as described in the Experimental section. Other details are as for Fig. 3.

^{125}I-hANF binding to cell-surface receptors on BAECs

Cell-surface-bound ^{125}I-hANF was quantified by extracting the ^{125}I-hANF from its receptor using 0.2 M-acetic acid/0.5 M-NaCl for 6 min at 4 °C (Haigler *et al.*, 1980; Hirata *et al.*, 1985; Johnson *et al.*, 1989). Non-specific binding of ^{125}I-hANF (in the presence of excess unlabelled hANF) constituted approx. 10–15% of total binding. The binding of 200 pM-^{125}I-hANF to cell-surface receptors on BAECs at 37 °C was very rapid, and within 2 min had achieved 73, 99% and 93% of the maximum value attained for control, NH_4Cl-treated and chloroquine-treated cells respectively (Fig. 6). Fig. 6 demonstrates that the cells rapidly achieve a roughly steady-state amount of cell-

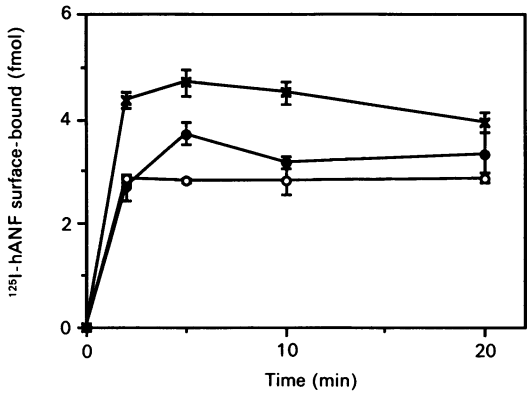

Fig. 6. Specific binding of 200 pM-^{125}I-hANF to the surface of cultured BAECs at 37 °C

^{125}I-hANF (200 fmol/well) was added to the confluent cell monolayers, and specific surface binding for control wells (●), wells preincubated in 20 mM-NH_4Cl (○) and wells preincubated in 0.2 mM-chloroquine (×) was quantified as described in the Experimental section. Results are means ± S.D. of three determinations.

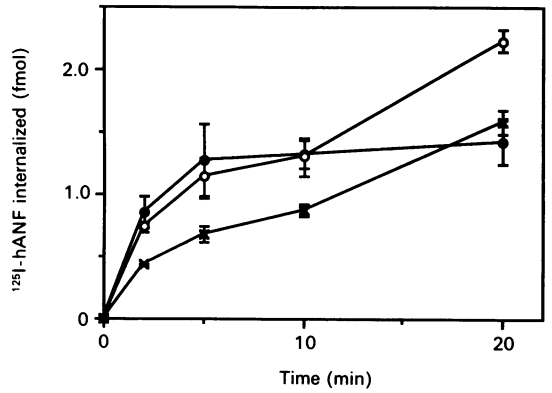

Fig. 7. Specific internalization of 200 pM-^{125}I-hANF by cultured BAECs at 37 °C

^{125}I-hANF (200 fmol/well) was added to the confluent cell monolayers, and specific internalization for control wells (●), wells preincubated in 20 mM-NH_4Cl (○) and wells preincubated in 0.2 mM-chloroquine (×) was quantified as described in the Experimental section. Results are means ± S.D. of three determinations.

surface-bound ^{125}I-hANF, and this steady state is essentially maintained for the duration of the experiment. Preincubation of the cells in 0.2 mM-chloroquine increased the quantity of surface-bound ^{125}I-hANF relative to control values and values obtained using cells preincubated in 20 mM-NH_4Cl.

Receptor-mediated internalization of ^{125}I-hANF by BAECs

Internalized ^{125}I-hANF was quantified as the residual cell-bound radioactivity remaining after cell-surface-receptor-bound ^{125}I-hANF had been removed (extracted) (Haigler *et al.*, 1980; Hirata *et al.*, 1985; Johnson *et al.*, 1989). Non-specific internalized ^{125}I-hANF (in the presence of excess unlabelled hANF) represented approx. 20–30 % of total internalized ^{125}I-hANF. As Fig. 7 shows (●, control values), the amount of intracellular radiolabel increased in a time-dependent manner up to 5 min and then maintained a steady state for the duration of the experiment. Conversely, both NH_4Cl and chloroquine induce a time-dependent increase in the intracellular accumulation of radiolabel throughout the duration of the experiment. Preincubation of the cells in 0.2 mM-chloroquine resulted in less intracellular radiolabel relative to control values in the first 10 min, whereas values attained after preincubation in 20 mM-NH_4Cl were roughly the same as control values attained in the first 10 min.

Excess unlabelled hANF blocks the rapid production of ^{125}I-Y and ^{125}I-RY, but not that of ^{125}I-FRY

To test the hypothesis that BAECs rapidly generate ^{125}I-Y and ^{125}I-RY from ^{125}I-hANF by a receptor-mediated endocytotic intracellular degradative mechanism, the rapid degradation of ^{125}I-hANF was monitored in the presence of excess unlabelled hANF. The excess (300 nM) unlabelled hANF was added to the cells to block the binding of the 200 pM-^{125}I-hANF to its receptor and thus block any degradative process that is mediated by the endocytosis of the receptor–ligand complex. As Table 1 shows, the presence of excess unlabelled hANF completely blocked (100 % inhibition) the rapid production of ^{125}I-Y and ^{125}I-RY from ^{125}I-hANF in the first 5 min. Conversely, the excess unlabelled hANF did not completely block the generation of the *C*-terminal tripeptide, ^{125}I-FRY, from ^{125}I-hANF, and only inhibited its production by 49 % (Table 1). The inability to block the generation of ^{125}I-FRY with excess unlabelled hANF indicates that it is generated via an extracellular proteolytic event.

DISCUSSION

The endothelium plays a crucial role in the metabolism of vasoactive peptides (Said, 1982). The simultaneous analysis of

Table 1. Degradation of ^{125}I-hANF by BAECs in the presence of excess unlabelled hANF

BAECs were prepared as described in the Experimental section. The ^{125}I-hANF (200 pM) in 0.1 % BSA/DMEM was added to the BAECs at 37 °C in the absence or presence of excess (300 nM) unlabelled hANF. After 5 min, the medium was collected, acidified to pH 2 with trifluoroacetic acid and analysed by h.p.l.c. Results are means ± S.D. of determinations.

Degradation product	Product (fmol) generated: In the absence of hANF	In the presence of 300 nM-hANF	Inhibition (%)*
^{125}I-Y	17.8 ± 3.1	0	100
^{125}I-RY	26.9 ± 3.9	0	100
^{125}I-FRY	24.8 ± 2.3	12.6 ± 1.3	49

* Percentage inhibition of the generation of degradation products by excess unlabelled hANF was calculated relative to the generation of degradation products in the absence of unlabelled hANF.

cell-surface receptor binding, internalization and degradation of ^{125}I-hANF by cultured endothelial cells has yielded a reasonably clear picture of the metabolism of this hormone in a cell-culture model of the vascular endothelium. The results of this study indicate that cultured BAECs degrade ^{125}I-hANF by a receptor-mediated intracellular mechanism and also by an extracellular proteolytic mechanism. The degradation of ^{125}I-hANF was monitored in the absence or presence of NH_4Cl, chloroquine and excess unlabelled hANF so as to identify catabolic mechanisms that were mediated via the endocytosis of the receptor–ligand complex. Weak bases such as NH_4Cl and chloroquine are believed to diffuse into cells in their unprotonated form and accumulate in acidic intracellular compartments such as endosomes and/or lysosomes, where they become protonated and raise intravesicular pH (for a review, see Dean *et al.*, 1984). This increase in intravesicular pH can act to inhibit acid-dependent proteinases/peptidases within organelles such as lysosomes. There is also a large body of evidence indicating that weak bases inhibit the movement of receptors in cells (for a review, see Wileman *et al.*, 1985). The addition of excess unlabelled hANF was utilized to block binding of ^{125}I-hANF to its receptor and thereby block degradative processes that were mediated via endocytosis of the receptor–ligand complex. Clearly, the finding that the rapid generation of ^{125}I-Y and ^{125}I-RY from ^{125}I-hANF was inhibited by NH_4Cl, chloroquine and excess unlabelled hANF indicated that these degradation products were derived mostly from a receptor-mediated delivery and breakdown of ^{125}I-hANF at some intracellular site.

The generation of the *C*-terminal tripeptide, ^{125}I-FRY, was not inhibited by NH_4Cl and chloroquine, nor was its production completely blocked by excess unlabelled hANF. These observations demonstrate that the *C*-terminal tripeptide was generated by an extracellular proteolytic event and was not generated via a receptor-mediated endocytotic process. The fact that the amount of ^{125}I-FRY generated does not increase in a time-dependent fashion suggests that the tripeptide is being degraded to some extent at later time points. We have previously observed the extracellular generation of the *C*-terminal tripeptide from ^{125}I-hANF by cultured smooth-muscle cells (Johnson *et al.* 1989). A peptidase with this specificity is of particular interest, since the presence of the Phe^{124}-Arg^{125}-Tyr^{126} residues in ANF are necessary to generate cyclic GMP in cultured vascular smooth-muscle (Scarborough *et al.*, 1986) and endothelial cells (Leitman *et al.*, 1986). Endopeptidase-24.11 (enkephalinase) (Stephenson & Kenny, 1987) and atrial dipeptidyl carboxyhydrolase (Harris & Wilson, 1984; Soler & Harris, 1989) catalyse the hydrolysis of the Ser^{123}–Phe^{124} bond of ANF. These enzymes are not responsible for the activity that we observed, since inclusion of 100 μM-SCH 39370, an endopeptidase-24.11 inhibitor (Haslanger *et al.*, 1989) or of 10 μM-captopril, an atrial dipeptidyl carboxyhydrolase (and angiotensin-converting-enzyme) inhibitor, (Harris & Wilson, 1984) in the medium did not affect the generation of ^{125}I-FRY from ^{125}I-hANF by the endothelial cells (G. R. Johnson, L. Arik, B. J. R. Pitts & C. J. Foster, unpublished work). The cultured BAECs rapidly cleared ~ 93 fmol of ^{125}I-hANF from the extracellular medium in 5 min. Approx. 25 fmol of this was converted into the *C*-terminal tripeptide ^{125}I-FRY. Therefore, we estimate that approx. 27 and 73% of the degradation of ^{125}I-hANF in the first 5 min was due to an extracellular and intracellular pathway respectively.

Since we have already studied the metabolism of ^{125}I-hANF by cultured vascular smooth-muscle cells (Johnson *et al.*, 1989), it is important that we compare and contrast the ways cultured vascular smooth-muscle and cultured vascular endothelial cells process radiolabelled hANF. Both types of cells rapidly clear ^{125}I-hANF from the extracellular medium, produce the same degradation products and generate the *C*-terminal tripeptide via an extracellular proteolytic event. NH_4Cl, chloroquine and excess unlabelled hANF had a profound effect upon the rapid extracellular appearance of ^{125}I-Y and ^{125}I-RY by endothelial cells, but not by smooth-muscle cells. This indicates that endothelial cells produce these degradation products within the cell and release them to the extracellular medium, whereas smooth-muscle cells mostly produce these via extracellular proteolysis (aminopeptidase and carboxypeptidase action). In contrast with the situation in endothelial cells, most of the ^{125}I-hANF that is cleared from the extracellular medium by receptors on smooth-muscle cells remains bound on the surface of the cells.

It is well documented that amines and proton ionophores inhibit the receptor-mediated endocytosis of ligands by affecting the recycling of receptors between intracellular stores and the cell surface (Wileman *et al.*, 1985). The results of our experiments suggest that hANF receptors in endothelial cells recycle between intracellular stores and the cell surface, either in the presence and/or absence of ligand. The fact that NH_4Cl, chloroquine and excess unlabelled hANF inhibit an endocytotic/degradative process that occurs so rapidly (within minutes after the addition of ^{125}I-hANF) suggests the involvement of early endosomes, since receptor recycling via early endosomes occurs with half-times of less than 3 min (Ciechanover *et al.*, 1983; Klausner *et al.*, 1983; Townsend *et al.*, 1984), while half-times for the delivery of ligands from endosomes to lysosomes are often slow (30–60 min) (Wolkoff *et al.*, 1984; Wall & Hubbard, 1985; Dunn *et al.*, 1986).

In conclusion, cultured endothelial cells rapidly degrade ^{125}I-hANF into three predominant products derived from its *C*-terminal region, ^{125}I-Y, ^{125}I-RY and ^{125}I-FRY. Two of these products, ^{125}I-Y and ^{125}I-RY, are rapidly generated via a receptor-mediated endocytotic process and intracellular breakdown, whereas the third, ^{125}I-FRY, is produced by an extracellular proteolytic event. The rapid metabolism of ^{125}I-hANF in this cell-culture model of the endothelium may provide additional insight into the mechanisms by which hANF is rapidly metabolized *in vivo*.

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