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ENDOTHELINS RELEASE ^{51}Cr FROM CULTURED HUMAN CEREBROMICROVASCULAR ENDOTHELIUM

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SUMMARY. The effects of vasoactive peptides endothelins (ET-1, ET-2, ET-3, S6b, S6c) on release of ^{51}Cr, production of inositol triphosphate (IP_3), and release of arachidonic acid (AA) were examined in cultured microvascular endothelium derived from human brain (HBEC). ET-1 induced dose-dependent release of ^{51}Cr (EC_{50}= 7±2 nM), transient increase of IP_3 (EC_{50}= 0.67±0.09 nM), and sustained release of AA (EC_{50} = 59±7 nM) from HBEC. Under the same experimental conditions, viability of the cells was preserved (>97%) as assessed by exclusion of vital dye trypan blue and release of lactate dehydrogenase (LDH). Dexamethasone (1 μM) inhibited ET-1-induced AA release, whereas it was ineffective on ^{51}Cr release. Protein kinase C (PKC) inhibitor H7 (200 nM), calcium channel blocker verapamil (10 μM), or IP_3 receptor antagonist ryonidine (5 μM) reduced ET-1 (100 nM)-induced release of ^{51}Cr. These findings indicate that endothelins can induce an increase of HBEC permeability by a receptor-specific activation of PKC and intracellular calcium mobilization.

The unique morphological features of cerebral microvascular endothelium, the main constituent of the blood brain barrier (BBB), are manifested by tight junctions, lack of fenestrations, and low rate of pinocytotic activity, all of which are considered an ultrastructural correlate of the highly restrictive nature of the BBB (1). In many diseases that affect the brain, changes in vascular permeability are associated with an altered appearance of the endothelium (increase in the number of vesicular profiles, abnormal junctions) and formation of brain edema (2, 3). The BBB dysfunction(s) could occur upon the release or activation of mediator substances derived from the blood, CNS, or the endothelium itself (4).

Endothelins (ET-1, ET-2, ET-3), 21-amino acid peptides originally isolated from the conditioned medium of cultured porcine vascular endothelium (5), are potent vasoactive substances which may be involved in pathogenesis of hypertension (6) and various cerebrovascular disorders [e.g. subarachnoidal haemorrhage (SAH), stroke] (7). Production and/or secretion of ET-1, as well as expression of endothelin receptors

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(ET_A, ET_B) have been demonstrated in cerebrovascular endothelial cells (8-10), various glial cells (11), and neurons (12). Non-vasoactive functions (i.e. mitogenic, neurosecretory, neuromodulatory) have also been described for endothelins in the brain (13-15). These functions are mediated through different second and third intracellular messengers including inositol phosphates (IPs) (16), Ca^{2+} (16, 17), protein kinase C (PKC), prostanoids (18), and adenylate cyclase (AC) (13).

Recently, we have described that HBEC secrete ET-1 in response to various vasoactive stimuli (agiotensin II, arginine-vasopressin) (8), and express receptors for ET-1 (18). This study demonstrates for the first time that endothelins may influence permeability of HBEC (i.e. ^{51}Cr release) through sequential activation of both phospholipase C (PLC) and phospholipase A_2 (PLA_2).

MATERIAL AND METHODS

Chemicals. [^{51}Cr]sodium chromate, [^{3}H]myoinositol, and [^{3}H]arachidonic acid were obtained from New England Nuclear (Boston, MA). Endothelin-1 (human/porcine), endothelin-2 (human), endothelin-3 (human/rat), sarafotoxin S6b, and sarafotoxin S6c were purchased from Sigma (St. Louis, MO). Antibodies to human factor VIII-related antigen and GFAP, and secondary antibodies were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY).

Cell Culture. HBEC were derived from small samples of temporal lobe removed surgically for the treatment of idiopathic epilepsy. Isolation and cultivation of microvessels (<50 μM) was performed by mechanical dispersion and filtration using a modified method of Gerhard et al. (1988) (19). Endothelial origin and purity (>98%) of HBEC cultures were assesed by positive immunocytochemical staining for von Willebrand (Factor VIII)-related antigen, incorporation of acetylated low-density lipoprotein (Dil-Ac-LDL), and negative staining for glial fibrillary acidic protein (GFAP). Propagated (passages 5-12), confluent cell cultures derived from three different cell lines were used in this study. All drugs used in indicated experiments were added to HBEC cultures 30 min prior to the addition of endothelins.

Release of ^{51}Cr and Lactate Dehydrogenase (LDH). Confluent HBEC grown in 96-microtiter plates were prelabeled with 0.3 μCi/well of ^{51}Cr-sodium chromate overnight at 37°C under 5% CO_2 in M199 (Gibco) containing 1% of human serum. Cells were washed, preincubated for 1 hr in M199, and treated with endothelins after medium replacement. Supernatants and cells (disrupted by overnight treatment with 1% Triton X-100) were counted for 1 min in a gamma counter (energy window set: 165-172 keV). Labeling efficiency for ^{51}Cr was 15-17%. The viability of the labeled cells was >97% as determined by the exclusion of the vital stain trypan blue. Loss of ^{51}Cr was expressed as percent of total activity (supernatant*100/cells+supernatant). LDH activity in both supernatants and cells was measured by the enzymatic method of Lowry and Passonneau (1974) (20).

Inositol Phosphates Assay. Inositol triphosphate (IP_3) formation was determined in the presence of 10 mM LiCl in cells prelabeled with [^{3}H]myoinositol (2.5 μCi/ml, 24 hrs). The formed inositol triphosphate was extracted with trichloroacetic acid (0.3 M), separated by anion exchange chromatography (Dowex AG1X8) and quantified as described previously (21). Protein content in the samples was assayed according to Lowry et al. (1951) (22).

Arachidonic Acid Release. [^{3}H]arachidonic acid (AA) release was determined in the HBEC prelabeled with [^{3}H]-arachidonic acid (0.5 μCi, 2 hrs; 58% incorporation), thoroughly washed with physiological buffered saline (PBS) three times at intervals of 5 min, and treated with various endothelins (2 min - 4 hrs). The radioactivity released into the medium was quantified by scintilation spectroscopy and expressed as a percent of the total activity (supernatants*100/cells + supernatants). Accordingly, the term "AA release" used in this study comprises both [^{3}H]-AA, and all labeled products of the AA metabolic cascade.

RESULTS

Effects of Endothelins on ^{51}Cr and LDH Release from HBEC. ET-1 induced a moderate release of ^{51}Cr from prelabeled HBEC starting at 0.1 nM, and reaching maximum at 100 nM (EC_{50} = 7 ± 2 nM) (Figure 1). Maximal release of ^{51}Cr was seen between 15 min and 1 hr after addition of ET-1 (results not shown). LDH release was not influenced by ET-1 (10 nM-1 μM) (Figure 1). Potency of various endothelins (at 10 nM and 250 nM) to induce ^{51}Cr release from HBEC was ET-1>ET-2>S6b>ET-3>S6c.

Effects of Endothelins on IP_3 Production and AA Release in HBEC. ET-1 dose-dependently stimulated both IP_3 production and AA release in HBEC (Figure 2). Concentration of ET-1 needed for half maximal stimulation of IP_3 (EC_{50}) was 0.67±0.09 nM, whereas it was 59±7 nM for AA release. Saturating concentrations of ET-1 for IP_3 stimulation and AA release were 10 nM, and 500 nM, respectively. Various endothelins stimulated both IP_3 production and AA release with similar relative efficacy: ET-1>ET-2>S6b>ET-3, while S6c was virtually ineffective on both parameters (Figure

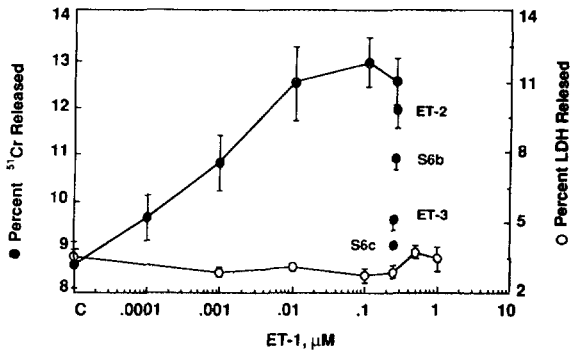

Figure 1. Dose-response of ^{51}Cr and LDH release from HBEC to ET-1. Both ^{51}Cr and LDH release were measured in the same experiment 1 hour after addition of the designated concentrations of ET-1 as described in Materials and Methods. ^{51}Cr release induced by other endothelins is presented at the saturating concentration (250 nM). Each curve depicts the data of a representative of three experiments (each point is Mean ± SEM of six replicates) showing similar results. Statistically significant release of ^{51}Cr ($p<0.05$; ANOVA) was observed starting at 0.1 nM of ET-1.

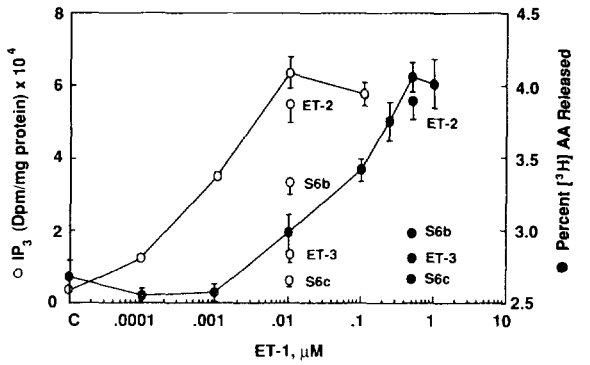

Figure 2. Dose-response of IP_3 formation and AA release to ET-1 in HBEC. IP_3 was measured at 15 min, whereas AA release was measured at 1 hour after addition of designated concentrations of ET-1. Responses to other endothelins (ET-2, ET-3, S6b, S6c) are presented at the saturating concentration for each parameter. Each curve depicts the data of a representative of three experiments (each point is Mean ± SEM of triplicate determinations) showing similar results. Statistically significant increase of IP_3 ($p<0.01$; ANOVA) was observed initially at 0.1 nM of ET-1, whereas AA release increased significantly ($p<0.01$; ANOVA) at 10 nM of ET-1. Estimated EC_{50} (ET-1) were (0.67 ± 0.09) nM and (59 ± 7) nM for IP_3 and AA-release, respectively.

2). Maximal accumulation of IP_3 was seen 15 min after addition of ET-1, while release of AA acid increased (relative to corresponding controls) over the period of 4 hours (Figure 3). However, the maximal rate (i.e. slope) of IP_3 formation was detected in the first minute, whereas maximal rate of AA release was observed between 5 and 15 min of ET-1 addition (Figure 3).

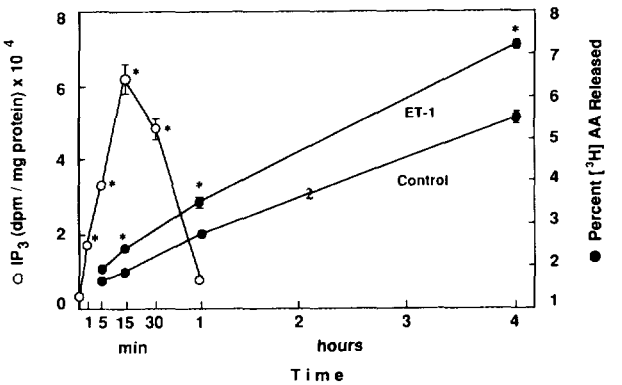

Figure 3. Time course of ET-1-induced IP_3 formation and AA release in HBEC. Maximal accumulation of IP_3 (ET-1 - 10 nM) was seen at 15 min; the maximal rate of IP_3 (dpm/min) was observed over the first minute of stimulation. ET-1 (100 nM)-induced AA accumulation increased over the period of 4 hours as compared to the corresponding controls; the rate of AA release was maximal between 5 and 15 min of stimulation. Each curve represents the data of a representative of three experiments (each point is Mean ± SEM of triplicate determinations) showing similar results.
*-indicates significant difference ($p<0.01$; Student's t-test) from corresponding control.

Effects of Drugs on ET-1-Induced IP_3, AA- and ^{51}Cr- Release. Pretreatment of HBEC with dexamethasone (1 μM) inhibited ET-1-induced AA release, but did not affect ET-1-induced IP_3 formation and ^{51}Cr release (Table 1). Phorbol-myristate ester (PMA; 10 μM) inhibited ET-1-induced IP_3 formation and potentiated AA release. In contrast, H7 (200 nM) prevented ET-1-induced ^{51}Cr release. Verapamil (10 μM) slightly reduced ET-1-stimulated IP_3 formation, AA and ^{51}Cr release from HBEC. Ryonidine (5 μM) also diminished both ET-1-stimulated AA and ^{51}Cr release (Table 1).

DISCUSSION

This study demonstrates that endothelins can influence the permeability (i.e. ^{51}Cr release) of human cerebromicrovascular endothelial cells. BBB permeability can be affected by a variety of blood-borne products (kinins, complement, bradykinin), mediators derived from blood cells (histamine, serotonin, leukotrienes, cytokines), tissue (eicosanoids, free radicals, polyamines), an cerebral endothelium itself (arachidonic acid and its metabolites) (1-4). Intracellular events which precede disruption of

Table 1. Effects of various drugs on ET-1-induced IP_3 production, AA release and ^{51}Cr release from HBEC

	IP_3 (dpm/mg protein)	AA (Percent of total)	^{51}Cr (Percent of total)
M199	3129 ± 123 (9)	3.08 ± 0.07 (9)	8.65 ± 0.49 (12)
ET-1 (100 nM)	52800 ± 2345 (9)	4.58 ± 0.02 (9)	12.74 ± 0.52 (12)
Dxm (1 μM)	3016 ± 150 (6)	2.88 ± 0.17 (9)	8.91 ± 0.51 (12)
ET-1 + Dxm	51720 ± 3247 (6)	3.19 ± 0.13 (9)*	12.06 ± 0.78 (12)
PMA (10 μM)	2884 ± 187 (9)	3.12 ± 0.12 (9)	11.65 ± 0.53 (12)†
ET-1 + PMA	25617 ± 1234 (9)*	5.09 ± 0.10 (9)*	11.97 ± 0.33 (12)
H7 (200 nM)	2640 ± 165 (6)†	3.27 ± 0.08 (9)	8.18 ± 0.58 (12)
ET-1 + H7	53121 ± 3214 (6)	4.52 ± 0.11 (9)	8.65 ± 0.44 (12)*
Verapamil (10 μM)	2998 ± 167 (9)	2.89 ± 0.12 (9)	6.89 ± 0.57 (12)
ET-1 + Verapamil	41824 ± 3121 (9)*	3.92 ± 0.09 (9)*	9.90 ± 0.63 (12)*
Ryonidine (5 μM)	3321 ± 243 (9)	3.00 ± 0.07 (9)	7.98 ± 0.61 (12)
ET-1 + Ryonidine	54678 ± 4321 (9)	4.01 ± 0.11 (9)*	10.61 ± 0.49 (12)*

Values are given as Means ± SEM for the number of replicates given in the parentheses. IP_3 formation was measured 15 min, whereas AA release and ^{51}Cr release were measured 1 hour after addition of ET-1.
† - indicates significant difference from M199 alone ($p<0.01$; Student's t-test).
* - indicates significant difference from ET-1 alone ($p<0.01$; Student's t-test).

cerebral endothelial integrity are presently not well understood. Exogenously added AA as well as generators of free radicals (H_2O_2, xanthine/xanthine oxidase) increase the permeability of HBEC for trypan blue-albumin complex and ^{51}Cr, and alter endothelial membrane fluidity (23). In addition, HBEC were shown to release various vasoactive products of AA metabolism (18, 24).

The role of different intracellular signal transduction processes (activation of PLC, PLA_2, PKC, AC, and changes in intracellular Ca^{2+}) in controlling endothelial permeability has been demonstrated for peripheral vascular beds (1, 4). Chopra et al. (25) have reported that among several markers (i.e. ^{111}In, LDH, ^{51}Cr) for peripheral endothelial cell injury the loss of ^{51}Cr from prelabeled cells was the most sensitive. ^{51}Cr in the cells is associated with low molecular weight cytosolic components of less than 1000 daltons, and may be released from the cells without structural disorganization of cell membrane (26). In contrast, larger molecules such as LDH (134,000 daltons), which is commonly used as a marker for endothelial cell injury, may be retained in the cells despite appeciable ultrastructural membrane derrangement (25). In addition, many biochemical alterations (i.e. increased prostaglandin production and impaired ATP metabolism) occur in "activated" EC before appreciable levels of ^{51}Cr release (25).

Endothelins have been implicated in pathogenesis of various cerebrovascular disorders associated with the BBB alterations, such as SAH and stroke (6, 7). We have previously shown that HBEC secrete immunoreactive ET-1 constitutively, and in response to various vasoactive peptides (angiotensin II, arinine-vasopressin), as well as to PKC activator PMA, and calcium ionophore A23187 (8). These cells also express specific receptors for endothelins (ET_A, ET_B) (18). High concentrations (>100 nM) of ET-1 are not cytotoxic to bovine aortic endothelial cells (27), but they can induce LDH release from cultured myocytes under hypoxic conditions (28). High doses of ET-1 (up to 1 μM) did not affect LDH release from HBEC.

^{51}Cr release from HBEC, and activation of both PLC and PLA_2 by ET-1 were induced upon the occupation of the same type of receptor, since the relative efficacy of various endothelins to induce ^{51}Cr release, IP_3 accumulation, and AA release were similar, and consistent with the described properties of ET_A receptor (10, 11). However, endothelin-induced IP_3 accumulation and AA release from HBEC appear to be largely independent of each other, since the time-course and EC_{50} values were considerably different. The dissociation of ET-1-induced IP_3 formation (inhibition) and AA release (stimulation) by PKC activator PMA in HBEC, further indicates that the PLC and PLA_2 are activated by different mechanisms.

However, it is not completely clear which ET-1- activated mediator system (IP_3-DAG-PKC, and/or AA cascade) is primarily responsible for the observed ET-1-induced permeability changes in HBEC. PKC inhibitor, H7, efficiently blocked ET-1-induced ^{51}Cr release from HBEC, suggesting that it is most likely related to diacyl-glycerol (DAG)-mediated activation of PKC. This is supported by the observations that PMA alone increased ^{51}Cr release (to the levels comparable to those seen with ET-1), and that ET-1 increased ^{51}Cr release from HBEC even when AA release was blocked by dexamethasone. Both ET-1-induced AA and ^{51}Cr release were diminished by ryonidine, an antagonist of intracellular IP_3 receptors and inhibitor of calcium mobilization from intracellular stores, as well as by verapamil, voltage-dependent Ca-channel blocker, implicating a two-step [$(Ca^{2+})i$] increment partly responsible for the activation of both PLA_2, and ^{51}Cr release.

The demonstrated endothelin-mediated increase of ^{51}Cr release from HBEC may signify subtle membrane changes allowing the loss of low molecular weight molecules. These findings suggest that excessive release of ET-1 may contribute to the alterations of BBB permeability observed during the course of cerebrovascular diseases such as hypertension and stroke.

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