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Endothelin-induced intracellular Ca^{2+} mobilization through its specific receptors in murine peritoneal macrophages

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We studied the presence of specific binding sites for endothelin (ET) and the effect of ET on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in murine thioglycolate-activated peritoneal macrophages. Scatchard analysis for binding experiments using [^{125}I]ET-1 or [^{125}I]ET-3 revealed the existence of a single class of binding sites. The binding parameters (K_d and B_{max}) for [^{125}I]ET-1 were almost identical to those for [^{125}I]ET-3. In addition, unlabeled 3 ET isopeptides (ET-1, ET-2 and ET-3) inhibited the specific binding of both ET-1 and ET-3 with similar inhibitory potencies. All 3 ET isopeptides caused an increase in $[Ca^{2+}]_i$ in the same dose-dependent manner (0.01–100 nM). These results demonstrate the existence of an ET receptor with the same affinity for all isoforms that mediates the ET-induced intracellular Ca^{2+} mobilization in murine peritoneal macrophages.

Endothelin; Receptor binding; Cytosolic free Ca^{2+}; Peritoneal macrophages; Mouse

1. INTRODUCTION

Endothelin (ET), a 21-amino acid peptide isolated from cultured endothelial cells, was originally identified as a strong vasoconstrictor [1]. Gene cloning and sequence analysis revealed the presence of 3 isoforms of the ET family peptides (ET-1, ET-2 and ET-3) in several mammalian species [2,3]. Furthermore, substantial biochemical and pharmacological studies in vivo and in vitro have elucidated that these ET family peptides possess a variety of effects in various tissues and cell types [4–6]. In particular, ET has been regarded as one of the regulatory factors in inflammatory responses occurring in microvasculature [5,7] and lung [8], because of its vasoconstrictive [1,5] and bronchoconstrictive [9,10] properties. However, the precise physiological and pathophysiological effects of ET in inflammatory responses remain to be clarified yet.

In the present study, we examined the effects of ET on mouse activated macrophages, since this cell type is known as a source of various chemical mediators involved in inflammation. First, we identified and characterized the specific receptors for ET on these cells using a ligand-binding technique with [^{125}I]ET-1 and [^{125}I]ET-3. Next, we studied the effect of ET on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells.

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Abbreviations: ET, endothelin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration

2. MATERIALS AND METHODS

2.1. *Isolation of murine thioglycolate-activated peritoneal macrophages and alveolar macrophages*

Peritoneal macrophages were collected from male C3H/HeN mice, injected with 2 ml of 3% thioglycolate broth intraperitoneally (i.p.) 4 days before the preparation, by peritoneal lavage with Modified Eagle's Medium (MEM) containing 2 U/ml heparin. After several washings, the cells were resuspended in RPMI 1640 medium and then plated out on culture dishes and incubated for 30 min at 37°C in a humidified atmosphere of 5% CO_2 and 95% air to allow adherence.

Alveolar macrophages were also collected from mice by the lung lavage technique as described by Holt [11]. The cell suspensions were plated onto culture dishes and incubated for 90 min at 37°C in 5% CO_2 and 95% air to allow adherence.

2.2. *Binding experiments*

The binding experiments were performed using the adherent cells (2×10^6 cells per 24-mm-diameter dish). Cells were incubated with 0.5 nM [^{125}I]ET-1 or [^{125}I]ET-3 in a total volume of 1 ml of Hanks' balanced salt solution (pH 7.4) containing 0.1% bovine serum albumin at 24°C for 90 min. After the incubation, the cells were washed with ice-cold 0.9% NaCl 3 times, and then solubilized in 1 N NaOH. The cell-bound radioactivity was measured using a gamma counter. Specific binding was determined by subtracting the non-specific binding, the amount of [^{125}I]ET-1 or [^{125}I]ET-3 bound in the presence of 100 nM unlabeled ET-1 or ET-3, from the total binding.

2.3. *Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)*

The adherent cells were harvested by treatment with 2.5 mM EGTA/0.9% NaCl for 15 min. Then the suspended cells were incubated with 5 μM fura-2 acetoxymethyl ester in RPMI 1640 medium at 24°C for 2 h. The fluorescence of signal of fura-2 of the suspended cells (1×10^6 cells/ml) was monitored while stirring at 37°C with CAF-100 Ca^{2+} analyzer (Japan Spectroscopic Co., Tokyo). The Ca^{2+} concentration was calculated as described elsewhere [12].

2.4. *Materials*

Synthetic ET-1, ET-2, ET-3 and big-ET were purchased from the Peptide Institute Inc. Osaka, Japan. [^{125}I]ET-1 and [^{125}I]ET-3 (respective specific activity 74 TBq/mmol) were obtained from Du

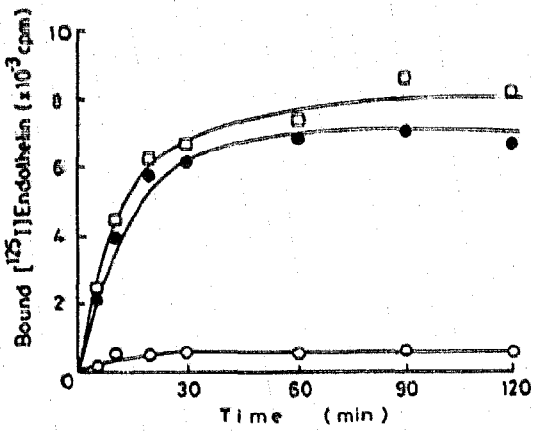

Fig. 1. Time course of association of [^{125}I]ET-1 to murine peritoneal macrophages. The cells (2×10^6 cell/well) were incubated with 0.5 nM [^{125}I]ET-1 at 24°C at the indicated times. Total binding (□), non-specific binding (○) and specific binding (•) were obtained as described in section 2. Each point is the mean value of duplicate determinations of two independent experiments.

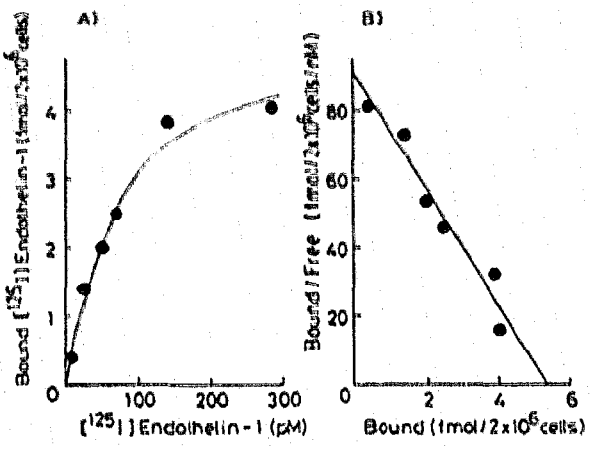

Fig. 2. (A) The specific binding of [^{125}I]ET-1 to murine peritoneal macrophages. The cells were incubated for 90 min at 24°C with different concentrations of [^{125}I]ET-1 in the presence or absence of 100 nM unlabeled ET-1. (B) Scatchard plot of the equilibrium binding data of (A). The data represent the mean value of 3 separate experiments done in duplicate.

Pont-New England Nuclear. Fura-2 acetoxymethyl ester was from Dojin Chemical, Kumamoto, Japan.

3. RESULTS

The specific binding sites for ET in murine peritoneal macrophages were examined using [^{125}I]ET-1 and [^{125}I]ET-3 as radioligands. As shown in Fig. 1, the specific binding of [^{125}I]ET-1 increased in a time-dependent manner and equilibrium levels were reached at 60 min. The specific binding at 24°C was about 90% of the total binding. Studies on the saturation binding to peritoneal macrophages revealed that the specific binding of [^{125}I]ET-1 exhibited complete saturability (Fig. 2A). Scatchard analysis of these data indicated the existence of a single class of binding sites (Fig. 2B), with an equilibrium dissociation constant (K_d) of 51.3 ± 5.8 pM and a binding capacity (B_{max}) of 4.9 ± 0.3 fmol/2 × 10^6 cells, equal to approximately 1464 ± 189 binding sites per cell. The binding kinetics of the saturation binding of [^{125}I]ET-3 was found to be almost the same with those of [^{125}I]ET-1 (summarized in Table I). In contrast, the specific bindings of [^{125}I]ET-1 as well as [^{125}I]ET-3 could not be significantly detected in mouse alveolar macrophages (data not shown).

Next, several ET isopeptides were examined for their abilities to compete with the specific binding of [^{125}I]ET-1 and [^{125}I]ET-3. Three unlabeled isopeptides inhibited [^{125}I]ET-1 and [^{125}I]ET-3 binding in a concentration-dependent manner (Fig. 3). All 3 isopeptides completely blocked both specific bindings at 10^{-7} M, whereas big-ET (proET) slightly inhibited at concentrations higher than 100 nM and other vasoactive peptides such as bradykinin, angiotensin II and arginine-vasopressin did not compete with the binding even at 10^{-6} M (data not shown). The rank order of inhibitory potencies for the isopeptides against the [^{125}I]ET-1 and [^{125}I]ET-3 bindings was the same (ET-1 = ET-2 ≥ ET-3).

The effect of ET on intracellular Ca^{2+} concentration was examined using fura-2-loaded macrophages. The transient and monophasic increase in $[Ca^{2+}]_i$ was observed during stimulation with ET. All 3 ET isopeptides caused an increase in $[Ca^{2+}]_i$ with the almost similar concentration-dependent manner (Fig. 4). On the other hand, big-ET (proET) caused only a slight increase in $[Ca^{2+}]_i$ (25% of the maximum) at concentrations higher than 100 nM.

Table I

Comparison of K_d and B_{max} values between [^{125}I]ET-1 and [^{125}I]ET-3 by Scatchard analysis

Ligand	K_d (pM)	B_{max} (sites/cell)
[^{125}I]ET-1	51.3 ± 5.8	1464 ± 109
[^{125}I]ET-3	59.0 ± 12.8	1206 ± 163

Data are man ± SE of 3 experiments.

4. DISCUSSION

In the present study, we provided the first evidence for the existence of specific receptors for ET in murine thioglycolate-activated peritoneal macrophages. Scatchard analysis revealed the same binding parameters between ET-1 and ET-3 to their specific binding sites (Table I). Furthermore, the displacement data (Fig. 3) indicated that all 3 ET isopeptides inhibited the specific binding of both ligands, and good correlation was observed between the rank orders of their inhibitory potencies against [^{125}I]ET-1 binding and those against

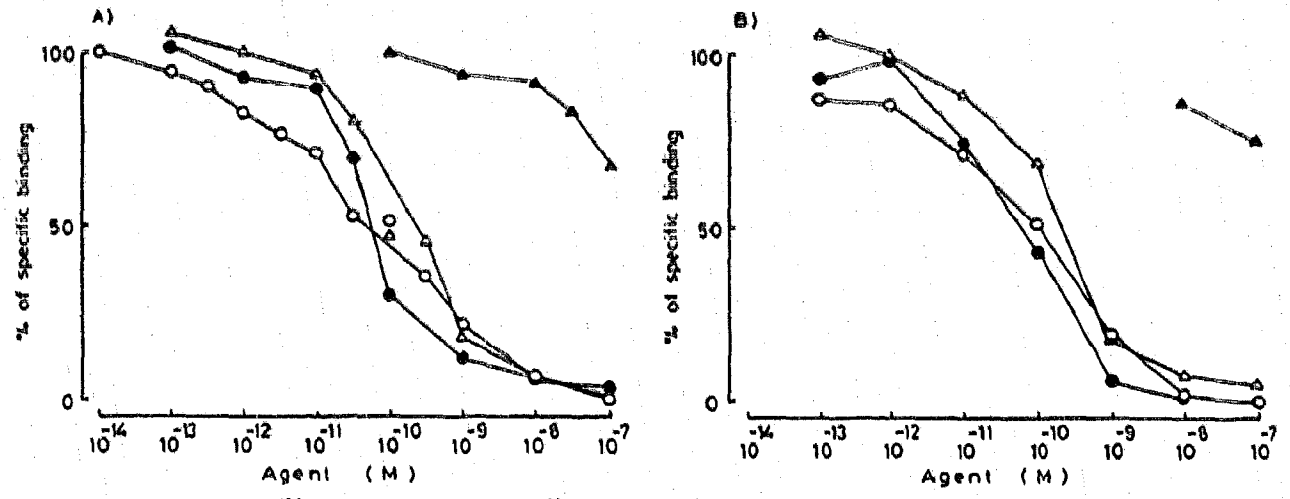

Fig. 3. Displacement of specific [^{125}I]ET-1 binding (A) and [^{125}I]ET-3 binding (B) to murine peritoneal macrophages by the ET isopeptides. The cells were incubated with 0.5 nM [^{125}I]ET-1 or [^{125}I]ET-3 in the presence of various concentrations of ET-1 (○), ET-2 (●), ET-3 (△) and big-ET (▲) at 24°C for 90 min. The control value (100%) was defined as the specific binding in the absence of these compounds. Each point is the average of the results of two independent experiments performed in duplicate.

[^{125}I]ET-3 binding. Recently, the existence of at least 3 receptor subtypes with different affinities for the ET isopeptides has been reported in a variety of tissues and cell types [13,14]. Our data demonstrate that ET receptors in mouse peritoneal macrophages belong to a less selective subtype that commonly recognizes 3 ET isopeptides with high affinity. This receptor subtype has also been detected in brain and kidney [14,15], although its physiological function still remains to be elucidated at present.

The present study also indicates that all 3 ET isopeptides concentration-dependently induced a transient increase in $[Ca^{2+}]_i$ in fura-2-loaded macrophages (Fig. 4). A lower selectivity for ET isopeptides highly correlated with the binding data, demonstrating the ET-induced intracellular Ca^{2+} mobilization to be elicited through specific receptors. In addition, depletion of external Ca^{2+} by treatment with 3 mM EGTA reduced Ca^{2+} mobilization to about 50% of the maximum (data not shown), suggesting Ca^{2+} mobilization from both intracellular as well as extracellular Ca^{2+} influx, as reported in vascular smooth muscle cells [16] and human mesangial cells [6,17].

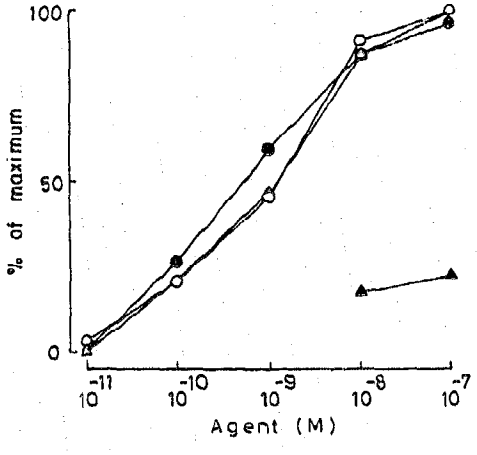

Fig. 4. Dose-dependency of the ET isopeptides in an increase in $[Ca^{2+}]_i$ in murine peritoneal macrophages. Macrophage suspensions (1.0×10^6 cells/ml) were stimulated with each ET isopeptide (0.01–100 nM) at 37°C. $[Ca^{2+}]_i$ was 108 nM in unstimulated macrophages, and maximal $[Ca^{2+}]_i$ (256 nM) was observed upon stimulation with 100 nM ET-1. The data are expressed as the percentage of the maximum increase in $[Ca^{2+}]_i$ (148 nM) referred to as 100%. Each point is the average of results of two independent experiments performed in duplicate.

Another important finding is the absence of the specific binding for both [^{125}I]ET-1 and [^{125}I]ET-3 in murine alveolar macrophages, although the specific binding sites were detected in peritoneal macrophages. The same results were also obtained in the rat (data not shown). Recently, the existence of tissue-specific distribution of ET receptor subtypes has been elucidated using ligand-binding as well as autoradiographical techniques [5]. Our results also demonstrate the tissue-specific expression of ET receptors in macrophages, which may be tightly associated with a diversity of ET's action.

Thus, ET may directly act on peritoneal macrophages through its specific receptors to modulate intracellular Ca^{2+} concentration. Recently, some pharmacological studies demonstrated that ET was induced by thrombin and the transforming growth factor (TGF-β), which are known to be produced in injured tissues [4], and that ET caused thromboxane A_2 release from isolated guinea-pig trachea and parenchyma [18]. Since the activated macrophages are known to contribute to tissue injury and produce a variety of chemical mediators involved in inflammatory responses [19], ET might modulate the inflammatory responses by affecting on the macrophages. We are now conducting further

studies to clarify the functional effects of ET on peritoneal macrophages.

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