PEPTIDES 29 (2008) 2250–2256

available at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/peptides

Regulation of glial inflammatory mediators synthesis: Possible role of endothelins

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form
7 September 2008
Accepted 8 September 2008
Published on line 16 September 2008

Keywords:
Endothelins
Prostaglandin E_2
Nitric oxide
Glial cells
Brain inflammation

ABSTRACT

Endothelins are well known as modulators of inflammation in the periphery, but little is known about their possible role in brain inflammation. Stimulation of astrocyte prostaglandin, an inflammatory mediator, synthesis was shown so far only by endothelin 3 (ET-3). By contrast, several studies showed no change or slight decrease of basal nitric oxide synthesis after treatment of astrocytes with endothelin 1 (ET-1) and ET-3. However, a significant increase in astrocytic and microglial nitric oxide synthase (NOS) was observed after exposure to ET-1 and ET-3 in a model of forebrain ischaemia. Here we demonstrate that all three endothelins (ET-1, ET-2, ET-3) significantly enhanced the synthesis of prostaglandin E_2 and nitric oxide in glial cells. Each of the selective antagonists for ETA and ETB receptors (BQ123 and BQ788 respectively), significantly inhibited endothelins-induced production of both nitric oxide and prostaglandin E_2. These results suggest a regulatory mechanism of endothelins, interacting with both endothelin receptors, on glial inflammation. Therefore, inhibition of endothelin receptors may have a therapeutic potential in pathological conditions of the brain, when an uncontrolled inflammatory response is involved.

1. Introduction

Compelling evidence derived from postmortem analysis of degenerative brains as well as from epidemiological studies of non-steroidal anti-inflammatory drug therapy [1] highlights the important role of inflammation in several degenerative diseases [26]. Both microglial cells and astrocytes, considered to be the main participants in this inflammatory process, demonstrate a reactive phenotype which is characterized, among other things, by the release of neuroinflammatory mediators such as nitric oxide, pro-inflammatory cytokines, and prostaglandins [3]. Of the many mediators thought to be involved in neuroinflammation, we concentrated on two, namely prostaglandins and nitric oxide. Prostaglandins are metabolites of arachidonic acid, produced by the sequential action of phospholipases and cyclooxygenase, and are considered to be critical mediators of both physiological and inflammatory processes in the brain [36]. Elevated levels of prostaglandins have been found in the cerebrospinal fluid of Alzheimer's disease patients and also in brains of patients following head trauma [11]. Nitric oxide is also considered to be involved in inflammatory process of neurodegenerative diseases, probably related to its free radical properties [5,29]. There is increasing evidence indicating that rising concentrations of brain nitric oxide during neuroinflammation accelerate progression of neurodegenerative diseases [19].

Several neuropeptides, produced by glial cells, have been shown to be involved in the regulation of brain inflammatory reactions. Previously we showed that bradykinin modulates glial inflammation [24]. In the present study we investigate the role of endothelins in mediating neuroinflammation. Endothelins, 21-amino acid vasoconstrictor peptides, are synthesized in a variety of tissues, including the brain (astrocytes, microglia and neurons) and are considered to possess neurotransmitter/

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2008.09.002

neuromediator and neuroendocrine actions [2]. At least three peptides, namely endothelin-1, -2 and -3, belong to the endothelin family. These peptides act via two distinct G-protein-coupled receptor subtypes, the endothelin A (ETA) and endothelin B (ETB) receptors. While ETB receptors equally bind all three endothelins, ETA receptors respond to only endothelin-1 and endothelin-2 [7]. Expression of ETA and ETB receptors was determined at the mRNA level by semi-quantitative reverse transcription polymerase chain reaction and at the protein levels in rat microglial cells [25]. Both these receptor subtypes are also highly expressed in astrocytes in culture in situ [34]. Differential activity of the receptors was reported by Danielyan et al. who showed that blockade of endothelin A but not B receptor protects astrocytes against hypoxic injury [6].

Nucci et al. were among the first groups to show that endothelin-1 stimulated the release of prostacyclin and thromboxane A2 from guinea pig or rat isolated lungs and nitric oxide in the perfused mesentery of rat [32]. Stimulation of astrocyte prostaglandin synthesis was shown so far only by endothelin 3 [21]. By contrast, several studies showed no change or slight decrease of basal nitric oxide synthesis after treatment of astrocytes with endothelin 1[33] and endothelin 3 or IRL 1620 (ETB receptor agonist), respectively [31]. These results were obtained with astrocyte-enriched cultures and not mixed glial cultures as used in the current study. However, a significant increase in glial nitric oxide synthase (NOS) was observed after exposure to ET-1 and ET-3 in a model of forebrain ischaemia [40]. As far as we know there is not any data regarding endothelin 2 effects on glial prostaglandin E_2 and nitric oxide release.

Several studies have shown that endothelins act as local pro-inflammatory factors in various inflammatory conditions both in the periphery and in the brain [22,12]. In addition, endothelins influence the release of neuroinflammatory mediators [28,33]. However, little is known about the role of endothelins in the regulation of prostaglandin E_2 and nitric oxide synthesis in glial cells. The present study was designed to investigate the role of endothelins in regulation of the synthesis of these mediators in glial cells and identify which receptors are involved.

2. Materials and methods

2.1. Primary mixed rat glial cell cultures

Primary glial cell cultures were prepared from whole brains of neonatal (24 h.) Wistar rats, according to well-established protocols [4]. Cells were grown to confluency in 24-well plates covered by poly-L-lysine at 37 °C in 8% CO_2 for 21 days. High glucose (4.5 mg glucose/ml) Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 0.2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.2 U/ml insulin, was used as the culture medium. The medium was replaced twice a week. Our previous immunocytochemistry studies revealed that these cultures contained about 70% astrocytes and 30% microglia at 18 days of culture [24].

Prior to an actual experiment, the cells were incubated in 1 ml serum-free medium for 16 h. The test agents were added to serum-free medium containing 0.1% bovine serum albumin and 10 mM HEPES which was used for incubation of the cells for a period of 24 h. At the end of each experiment, cells were harvested with a mixture of trypsin and serum-free medium (1:1), and counted using Z1 Coulter counter (Coulter Electronics, Miami, Florida, USA).

The following agents were used: Endothelin-1, Endothelin-2, Endothelin-3, BQ-123 (a selective ETA receptor antagonist), BQ-788 (a selective ETB receptor antagonist). All were purchased from Sigma–Aldrich, St. Louis, MO.

2.2. Determination of nitric oxide

Accumulation of nitric oxide in the medium was determined by measuring nitrite (a stable breakdown product of NO), using the Griess method as previously described [9,15]. A solution of $NaNO_2$ was used as standard. 100 μl of culture media supernatants or standards were incubated in the dark at room temperature for 15 min with equal amounts of Griess reagent in 96-well plates. Subsequently, absorbance was read at 540 nm using a microplate reader (model 680, Bio-Rad, Bath, UK).

2.3. Prostaglandin E_2 assay

Assessment of prostaglandin E_2 that had accumulated in the un-extracted samples of the medium was performed as described previously by our group [24], using a single antibody radioimmunoassay with dextran-coated charcoal precipitation. Samples were incubated with rabbit antibody to prostaglandin E_2 for 30 min. Precipitation of unbound prostaglandins was achieved by incubation of the samples with dextran-coated charcoal for 10 min and centrifugation at 2000 × *g*, at 4 °C for 15 min.

Radioactivity was measured in the resulting supernatant on β-scintillation counter (TriCarb 2100, Packard, Meriden, CT, USA). The sensitivity of the assay was 0.07 ng/ml. Rabbit antibody to prostaglandin E_2 was obtained from Sigma (Sigma–Aldrich, St. Louis, MO, USA), and tritium-labeled prostaglandin E_2 (160 Ci/mmol) was supplied by the Radiochemical Center (Amersham, UK).

2.4. Statistics

Results are expressed as means ± S.E.M. for each experiment. Statistical analysis of the results was performed using one way analysis of variance (ANOVA) followed by Tukey–Kramer (multiple comparisons post-test) test if the ANOVA's $P < 0.05$. Values of $P < 0.05$ of Tukey test were considered statistically significant.

3. Results

Endothelins-induced PGE_2 synthesis is mediated by ETA and ETB receptors:

As shown in Fig. 1A, endothelin 1 (ET-1) (10^{-9}–10^{-7} M) enhanced prostaglandin E_2 synthesis in glial cells in a concentration-dependent manner. Similar results were obtained with endothelin 2 (ET-2) (10^{-9}–10^{-7} M) (Fig. 1B) and endothelin 3 (ET-3) (10^{-9}–10^{-7} M) (Fig. 1C). While ETB receptors equally bind all three endothelins, ETA receptors respond to