ASTROCYTES AND CHRONIC PAIN MECHANISMS – THE ROLE OF HISTAMINE, IL-1β AND NGF

Metoda Lipnik-Stangelj1, Slavina Surcheva2, Ilonka Ferjan1, Mila Vlaskovska2
1University of Ljubljana, Faculty of Medicine, Ljubljana, Slovenia
2Medical University of Sofia, Faculty of Medicine, Sofia, Bulgaria
Correspondence to: Metoda Lipnik-Stangelj
E-mail: metoda.lipnik-stangelj@mf.uni-lj.si

ABSTRACT
In exaggerated pain states, the activated astrocytes release several signalling molecules involved in cellular mechanisms of chronic pain. Among them, nerve growth factor (NGF) and interleukin (IL)-1β are both recognized as potent algogens. We previously showed that histamine is a potent stimulator of NGF production in rat cortical astrocytes in primary culture. Since histamine and IL-1β have common interactions in different physiological responses, in the present work we were interested to elucidate the molecular mechanisms involved in the interactions between histamine, IL-1β and NGF that could contribute to the processing of chronic pain. As an experimental model we used cultured rat cortical astrocytes. NGF and IL-1β levels in the culture medium were measured by ELISA. IL-1β mRNA expression was determined by RT PCR.
The results showed that the co-treatment of the cultured astrocytes with histamine and IL-1β significantly increased NGF secretion in comparison to the secretion observed with either histamine, or IL-1β alone. The histamine and IL-1β effect on NGF secretion was additive, dose-dependent and increased with increased concentrations of either histamine, or IL-1β. The additive effect of histamine and IL-1β on NGF secretion was strongly suppressed by histamine H1 receptor antagonist/inverse agonist mepyramine, protein kinase C (PKC) inhibitors (GF 109203X, Go 6976), and mitogen-activated protein kinase kinase (MAPK)-1 inhibitor (PD 98059); however, they did not influence significantly the NGF secretion evoked by IL-1β alone. Histamine also stimulated the secretion of IL-1β from cultured astrocytes and induced higher expression of IL-1β mRNA in comparison to untreated cells.
We concluded that histamine potently interacts with the synthesis and secretion of IL-1β and NGF in astroglial cells, and therefore can contribute to the development and maintenance of chronic pain, mediated by both algogens.

Keywords: astrocytes, histamine, IL-1β, NGF, chronic pain
Abbreviations: CNS: central nerve system; HA: histamine; IL-1: interleukin-1; MAP kinase: mitogen-activated protein kinase; MEK: mitogen-activated protein kinase kinase; NGF: nerve growth factor; PKC: protein-kinase C; TPA: 12-O-tetradecanoylphorbol-13-acetate

Introduction
Astrocytes in the central nervous system play roles in many aspects of neuronal functioning, and recent studies confirmed their importance in pain processing. Peripheral tissue damage or inflammation initiates signals that alter the function of microglia and astrocytes as well, which in turn release factors that regulate the nociceptive neuronal excitability (6, 18). Glial cells, like immune cells, react not only at sites of central and/or peripheral nervous system damage, but also at sites remote from the focus of injury or disease. The microglial involvement in various pain states is well documented. There is also evidence for the involvement of astrocytes and their role may sometimes even be more dominant than that of microglia (2, 3, 7, 17, 20).

Astrocytes make close contacts with synapses and recent studies showed that astrocytes reaction after nerve injury, arthritis, and tumour growth is more persistent than microglial reaction, and displays a better correlation with chronic pain behaviours (6, 7, 18). In exaggerated pain states, reflected in hyperalgesia and allodynia, the activated astrocytes may release gliotransmitters such as glutamate (21), ATP, D-serine, or neurotrophic factors (i.e., nerve growth factor [NGF]), cytokines (i.e., interleukin [IL]-1β), chemokine chemo attractant ligands-2 and other signalling molecules involved in the cellular mechanisms of chronic pain (4, 7, 14, 18, 21). Among them, NGF and IL-1β are both recognized as potent algogens, both involved in a complex network which is able to enhance and prolong persistent pain states (9, 13, 22).

NGF-mediated effects are central to inflammatory, surgical, visceral and neuropathic pain (1, 9, 25, 26). In developing dorsal root ganglion neurons NGF stimulates local synthesis of cAMP-response-element-binding protein, leading to retrograde transport to the neural soma (5). NGF increases the activity of the P13 kinase/protein-kinase B pathway in sensory neurons (29) and potently activates the mechanistic target of the rapamycin (mTOR) signalling pathway to cap-dependent translational processes, which contributes to nociceptive plasticity (15).

IL-1β has been found as a major pro-inflammatory cytokine and is up-regulated in the spinal cord under different

METODA LIPNIK-STANGELJ

chronic pain conditions (7, 8, 16, 23). Up-regulation of IL-1β has been observed in astrocytes after bone cancer (28), nerve injury (24) and hind paw inflammation (27), and an important role of IL-1β for pain sensitization has been shown (6, 16, 27). Alleviation of inflammatory, neuropathic, and cancer pain has been demonstrated following inhibition of spinal IL-1β signalling with intrathecal IL-1 receptor antagonist or neutralizing antibody (6).

In our previous studies we showed that histamine is a potent stimulator of NGF production in rat cortical astrocytes in primary culture (10, 11, 12). Among cytokines, IL-1β also potently enhances NGF synthesis. Besides their stimulatory effect on NGF production, histamine and IL-1β have common interactions in different physiological responses (10). In the present work we looked into the molecular mechanisms involved in the interactions between histamine, IL-1β and NGF in rat cortical astrocytes in primary culture that could contribute to the processing of chronic pain.

Materials and Methods

Materials

L-15 Leibowitz medium, Foetal Bovine Serum (FBS), Dulbecco’s modified Eagle medium and Ham’s nutrient mixture F-12 (DMEM / F12), Penicillin–Streptomycin Dulbecco’s modified Eagle medium and Ham’s nutrient L-15 Leibowitz medium, Foetal Bovine Serum (FBS), Materials

Animals

Newborn Wistar rats (postnatal day 2) were obtained from our own breeding colony. The animals were maintained under constant environmental conditions, with an ambient temperature of 22 ± 1 °C, relative humidity of 55 ± 10 %, and a natural light–dark cycle. The breeding colony was kept in Ehret type-4 cages (Emmendingen, Germany). The bedding material was Lignocel 3/4. The colony received a standard rodent diet (Altromin, Lage, Germany), and had free access to food and water. We used four newborn animals for each experiment.

All the animal procedures were approved by the National Animal Ethical Committee of the Republic of Slovenia (licence number 323-02-232/2005/2) and were conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

Astrocytes culture preparation

Primary cultures of rat cortical astrocytes were prepared from the brain of new-born Wistar rats. The new-born rats (postnatal day 2) were decapitated and the brains removed aseptically. After removal of the meninges, the cortices were transferred to a Petri dish containing the L-15 (Leibowitz) medium. The cortices were then mechanically dissociated into 10 mL of culture medium, consisting of DMEM/F12 (1:1), FBS at a volume fraction of 10 %, 100 U/mL penicillin and 100 μg/mL streptomycin. The cell suspension was triturated and plated into tissue-culture flasks. The cells were grown at 37 °C in a water-saturated air environment containing 10 % CO₂. In order to purify the cultures, when they became confluent, they were shaken at 150 rpm for 18 h to remove microglial cells. After the shaking, the medium was changed and the cells were trypsinized and cultured for 24 h in the presence of 10 μmol/L cytosine arabinoside that only allowed the growth of astrocytes. After reaching confluence again, the cells were subcultured onto 35 mm Petri dishes for the treatment.

The purity of the culture was checked using immunocytochemical staining for the glial fibrillary acidic protein, which is the major component of the astrocyte cytoskeleton.

Treatment of the cells

After the cultures became confluent, the culture medium was replaced with 1 mL of fresh medium and the cells were treated as follows:

a) The cells were treated with different concentrations of histamine (0.1 mmol/L, 1 mmol/L, 10 mmol/L, 0.1 μmol/L, 1 μmol/L, 10 μmol/L) or different concentrations of IL-1β (0.1 ng/mL, 1 ng/mL, 10 ng/mL) for 24 h.

b) The cells were treated with different concentrations of histamine (0.1 μmol/L, 1 μmol/L, 10 μmol/L) in the presence of different concentrations of IL-1β 0.1 mg/mL, 1 mg/mL, 10 mg/mL) for 24 h.

c) The cells were pre-treated with 10 μmol/L mepyramine or antibody of IL-1β (abIL-1β) for 30 min. Then the cells were treated with 1 μmol/L of histamine in the presence of 1 mg/mL IL-1β for 24 h.

d) The cells were pre-treated with selective PKC inhibitors (1.0 μmol/L of GF 109203X or 0.1 μmol/L G06976) or MAP kinase inhibitor (10 μmol/L of PD 98059), for 30 min. After pre-incubation, the cells were treated with 1 μmol/L of histamine in the presence of 1 ng/mL IL-1β for 24 h.

e) The cells were treated with 1 μmol/L of histamine. Aliquots of 10 mg of the cells were stored in RNA...
Stabilization Reagent at -70 °C and used for RNA analysis.

The control cells remained untreated. The concentrations of mepyramine, PKC inhibitors, MAP kinase inhibitor, and abIL-1β used in our study were chosen from dose-response curves obtained in our previous studies (data not shown). The experiments were performed under lipopolysaccharide-free conditions.

After the treatment, the culture medium was collected to determine the levels of either NGF, or IL-1β. Cells from individual dishes were harvested for protein determination or used for RNA analysis.

Protein determination
The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard. The culture medium was collected, frozen and used for the NGF and IL-1β determinations.

Enzyme-linked immunoassay
The NGF levels in the culture medium were determined using NGF enzyme immunoassay, following the Boehringer Mannheim protocol. IL-1β levels in the culture medium were determined using IL-1β enzyme immunoassay, using Pierce Biotechnology IL-1β–ELISA protocol.

RNA analysis
Total RNA from three histamine-treated and three control samples was isolated, and treated with DNase. Then reverse transcription of total RNA to cDNA was done. To analyse IL-1β mRNA expression, quantitative real-time PCR was performed on ABI PRISM 7900HT Fast Real-Time PCR System, using TaqMan Gene Expression Assay. Eukaryotic 18S rRNA was used as an endogenous control. Each measurement was run in triplicate in a 10 μL reaction mixture, for IL-1β cDNA and 18S rRNA in separate wells. Quantification was done using the relative standard curve method. IL-1β mRNA expression was first calculated as the mean of the three experimental replicates relative to a normalization factor based on 18S rRNA expression in the same replicates. Relative expression results for histamine-treated samples were reported as a fold difference in their normalized IL-1β mRNA expression compared to normalized IL-1β mRNA expression of control samples, the latter being used as a calibrator.

Statistical analysis
Determinations of NGF and IL-1β release are shown as means ± standard error of the mean (SEM) of three independent assays. Statistical analysis of data was performed by one-way ANOVA with either Dunnett’s or Scheffe’s multiple comparison tests.

Results and Discussion
In a previous study we showed that histamine potently stimulates NGF secretion from cultured astrocytes by the activation of histamine H1 receptors and stimulation of PKC and MAP kinase (10). The results showed that the co-
treatment of the cultured astrocytes with both histamine and IL-1β significantly increased NGF secretion in comparison to the secretion observed following treatment with either histamine (HA), or IL-1β alone. The effect of histamine and IL-1β on NGF secretion was additive, dose-dependent and increased with increased concentrations of either HA, or IL-1β. The enhancement of NGF secretion became significant at concentrations of 100 nmol/L of histamine and 1 ng/mL IL-1β (Fig. 1).

Fig. 1. Stimulation of NGF secretion from cultured astrocytes by histamine (HA) and IL-1β after 24 h of incubation. The cells were co-treated with different concentrations of histamine and IL-1β for 24 h. The levels of NGF in the culture media were determined by NGF-ELISA Each value is the mean ± SEM of three independent experiments, each assayed in duplicate.

The presence of histamine H1 receptor antagonist/inverse agonist mepyramine at a concentration 10 μmol/L effectively blocked the stimulatory effect of histamine (1 μmol/L) on NGF release from cultured astrocytes, but did not modify the NGF secretion evoked by IL-1β. Similarly, the presence of abIL-1β effectively blocked the stimulatory effect of IL-1β (1 ng/mL) on NGF release from cultured astrocytes, but did not modify the NGF secretion evoked by 1 μmol/L histamine (Fig. 2).

Next, we looked into the signalling pathway involved in the interaction between histamine and IL-1β, where additive effect on NGF secretion was observed. The results showed that the additive effect of histamine and IL-1β on NGF secretion was strongly suppressed by PKC inhibitors (GF 109203X, Go
In the present study we showed that histamine is strongly involved in the interactions between two potent algogenes, IL-1β and NGF, in cultured astrocytes. While histamine stimulation of NGF secretion is mediated via activation of histamine H1 receptors and PKC-MAPK signalling pathway, the synthesis and secretion of NGF can be regulated via various receptors and molecular mechanisms which involve four main signalling pathways: activation of adenylate cyclase, phospholipase Cβ, phospholipase A2, and mobilisation of Ca2+ ions (10). Several substances, such as neurotransmitters, growth factors, cytokines and steroids participate in this process. In this sense, the NGF production can be amplified following treatment of the cells with histamine, serotonin, dopamine, adrenaline, isoproterenol, derivatives of 1,4-benzoquinone, 12-O-tetradecanoylphorbol-13-acetate (TPA), interleukin-4 and IL-5, and some growth factors [for review see (10)].

Besides histamine, IL-1β also possesses a strong stimulatory effect on NGF production. The NGF synthesis, evoked by IL-1β, is mediated via activation of IL-1β receptors. In the signal transmission, distinct protein kinases and phosphoprotein-phosphatases are involved. However, IL-1β can also generate a PKC cascade in certain transcription processes and acts synergistically with PKC-activators phorbol-esters in the stimulation of NGF synthesis and secretion (10, 19). We found that simultaneous treatment of the astroglial cells in primary culture with histamine and IL-1β significantly increased NGF secretion in comparison to the secretion observed following treatment with either histamine, or IL-1β alone (Fig. 1). The effect of histamine and IL-1β was dose-dependent and additive, and this closely correlates with findings, where TPA, which is also a potent stimulator of PKC, and IL-1β gave an additive increase in NGF mRNA content and NGF secretion from cultured astrocytes (19). TPA enhances the NGF synthesis through the PKC-stimulated activation of activator protein-1, which is also involved in the NGF synthesis evoked by histamine (10, 19).

Histamine H1 receptor antagonist/inverse agonist mepyramine effectively blocked the additive effect of histamine and IL-1β to the level of the stimulation of NGF secretion by IL-1β alone, but it had no effect on NGF secretion stimulated by IL-1β alone (Fig. 2). Similarly, PKC inhibitors (GF 109203X, which is selective for the conventional PKC isoform group, and Go 6976, which is Ca2+-dependent, PKC alpha isoform selective inhibitor), and MEK1 inhibitor (PD 98059) suppressed the additive effect of histamine and IL-1β, but failed to inhibit the stimulatory effect of IL-1β (Fig. 3). In accordance with these findings, down-regulation of PKC-activity also diminishes the additive effect of PKC-stimulator TPA and IL-1β, but does not influence the stimulatory effect of IL-1β (19).

These observations indicate that histamine and IL-1β stimulate NGF synthesis and secretion via different signalling pathways, however, stimulation of histamine H1 receptor and activation of PKC–MAPK/MEK1 signalling pathway is
crucial in the interaction between histamine and IL-1β, where additive effect occurs.

To examine the interaction between histamine and IL-1β, we also determined the influence of histamine on IL-1β synthesis and secretion. We found that histamine enhanced IL-1β secretion from cultured astrocytes after 24 h incubation. We also found enhanced expression of IL-1β mRNA in the cells treated with histamine. These results indicate that histamine strongly contributes to the network, which is able to enhance and prolong persistent pain states, mediated by IL-1β and/or NGF.

For the NGF production evoked by histamine, activation of the PKC–MAPK signalling pathway is crucial. This observation is important since recent studies showed that peripheral inflammation and nerve injury activate several signalling pathways (e.g., protein kinases A and C, calcium/calmodulin-dependent protein kinase) in primary sensory and dorsal horn neurons that then activate MAPKs, including p38 MAPK, extracellular signal-regulated kinase, and c-Jun N-terminal kinases in spinal and medullary dorsal horn neurons and microglia or astrocytes or both. Activation of MAPKs in further steps leads to the glial production of pro-inflammatory mediators (e.g., IL-1β) that sensitize dorsal horn neurons as well as induce behavioural hyperalgesia and allodynia. Recent studies have particularly emphasized the importance of chemokines in inflammatory and neuropathic pain states (2, 6, 7, 20).

Conclusions

Based on our results we concluded that histamine potently interacts with IL-1β and NGF in cultured astrocytes and therefore can contribute to the development and maintenance of chronic pain mediated by both algogens. Histamine stimulates IL-1β and NGF synthesis and secretion in cultured astrocytes. In addition, histamine and IL-1β exert additive effect on NGF secretion. Our findings contribute to the clarification of specific signalling pathways in astrocytes that may offer new approaches for the management of chronic pain.

Acknowledgements

The work was supported by research grants P3-0067 and SI-BG/2011-12/11 from the Ministry of Higher Education, Science and Technology, Republic of Slovenia, and DTNS/ BG-SL/2011 from the Ministry of Education, Youth and Science, Republic of Bulgaria.

REFERENCES