ELSEVIER

European Journal of Pharmacology 374 (1999) 309–313

ejp

www.elsevier.nl/locate/ejphar

Short communication

Functional expression of the fractalkine (CX_3C) receptor and its regulation by lipopolysaccharide in rat microglia

Erik W.G.M. Boddeke [a,*], Inge Meigel [b], Stefan Frentzel [b], Knut Biber [a], Li Q. Renn [c], Peter Gebicke-Härter [c]

[a] *Department of Medical Physiology, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, Netherlands*
[b] *Novartis Pharma, NS Research, CH4002 Basel, Switzerland*
[c] *Department of Psychiatry, University of Freiburg, 79104 Freiburg, Germany*

Received 19 April 1999; accepted 22 April 1999

Abstract

Functional expression of CX_3CR1, a recently discovered receptor for the chemokine fractalkine, was investigated in cultured rat microglia. Reverse transcriptase polymerase chain reaction (PCR) experiments show abundant expression of fractalkine receptor mRNA in microglia. mRNA expression of fractalkine was undetectable in astrocytes and microglia but was very strong in cortical neurons. Incubation of microglia with lipopolysaccharide (100 ng/ml) transiently suppressed expression of fractalkine receptor mRNA. Fractalkine induced a concentration-dependent (10^{-10}–10^{-8} M) and, at high concentrations, oscillatory mobilization of intracellular Ca^{2+} in microglia The concentration–response curve of fractalkine was shifted to the right after 12 h incubation with lipopolysaccharide. It is concluded that treatment with endotoxin downregulates expression of fractalkine receptor mRNA in rat microglia and suppresses the functional response to fractalkine.

Keywords: Fractalkine; CX_3C_1 receptor; Lipopolysaccharide

1. Introduction

Chemokines are small proteins that stimulate migration of leukocytes and mediate inflammation (Baggiolini, 1998). These proteins are classified according to characteristic cystein signature motifs into subgroups (Baggiolini, 1998). Recently, a chemokine bearing a new CX_3C cystein motif has been cloned (Bazan et al., 1997; Pan et al., 1997). This chemokine, named fractalkine, displays potent chemoattractant activity for T cells and monocytes and is of non-haemopoietic origin (Bazan et al., 1997). Fractalkine seems to be produced by endothelial cells and also by neurons and occurs as a cell surface-bound as well as a cleaved protein.

Recently, the orphan receptors V28 and RBS11 have been characterized as the human and rat receptors for fractalkine, respectively, and have been renamed CX_3CR1 (Imai et al., 1997; Jiang et al., 1998). The fractalkine receptor is a seven transmembrane-spanning G protein-coupled receptor expressed in leukocytes. Its signal transduction presumably plays a role in their migration and adhesion.

Both fractalkine and its receptor are constitutively expressed in human and rat brain, suggesting a physiological role of this chemokine in the nervous system. Recently, expression of fractalkine has been observed predominantly in neurons, whereas its receptor was primarily detectable in microglia cells and not in neurons (Nishiyori et al., 1998). Accordingly, it has been suggested that fractalkine acts as a paracrine signaling molecule from neuron to microglia (Harrison et al., 1998; Nishiyori et al., 1998). In this respect, the expression of fractalkine receptor mRNA may be regulated and thus involved in recruitment of leukocytes and microglia during neuroinflammation. Indeed an increase in fractalkine receptor mRNA in the facial nucleus has been observed after lesion of the rat facial nerve (Harrison et al., 1998). This increase, however, may be due to an increased number of activated microglia migrating into the lesion-area.

* Corresponding author. Tel.: +31-50-363-2701; Fax: +31-50-363-2751; E-mail: h.w.g.m.boddeke@med.rug.nl

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00307-6

In this study, we have investigated the regulation of the expression of mRNA encoding the fractalkine receptor and its intracellular calcium signaling by exposure to an inflammatory stimulus using bacterial lipopolysaccharide.

2. Materials and methods

2.1. RT-PCR analysis

Primary astrocyte and microglia cultures were prepared from male Sprague–Dawley rats as described previously (Gebicke-Härter et al., 1989). Enriched cortical neuronal cultures were prepared from male Sprague–Dawley rats as described by Spleiss et al. (1998). Cells were lysed in guanidinium isothiocyanate/mercaptoethanol solution and total RNA was extracted according to Chomczynski and Sacchi (1987). 1 μg total RNA was reverse-transcribed into cDNA. Potential contamination by genomic DNA was checked by running the reactions without reverse transcriptase and using S12 primers in subsequent PCR amplifications. PCR amplifications were performed as described (Spleiss et al., 1998). In all experiments 35 cycles have been used.

The following primer pairs were used:

fractalkine	# 194: TGGAGACGACACAGCACAG
	# 946: TGGCATGGATGGGTTCCTC
fractalkine receptor	# 1432: TCTTCTGGACGCCTTACAACATCG
	# 1728: TCAAGCTGCTCAGAATGCTGTCCT
S12	# 49: ACGTCAACACTGCTCTACA
	# 360: CTTTGCCATAGTCCTTAAC

Accession numbers of cDNAs, where primers have been deduced from are: fractalkine murine: Acc.# U92565;

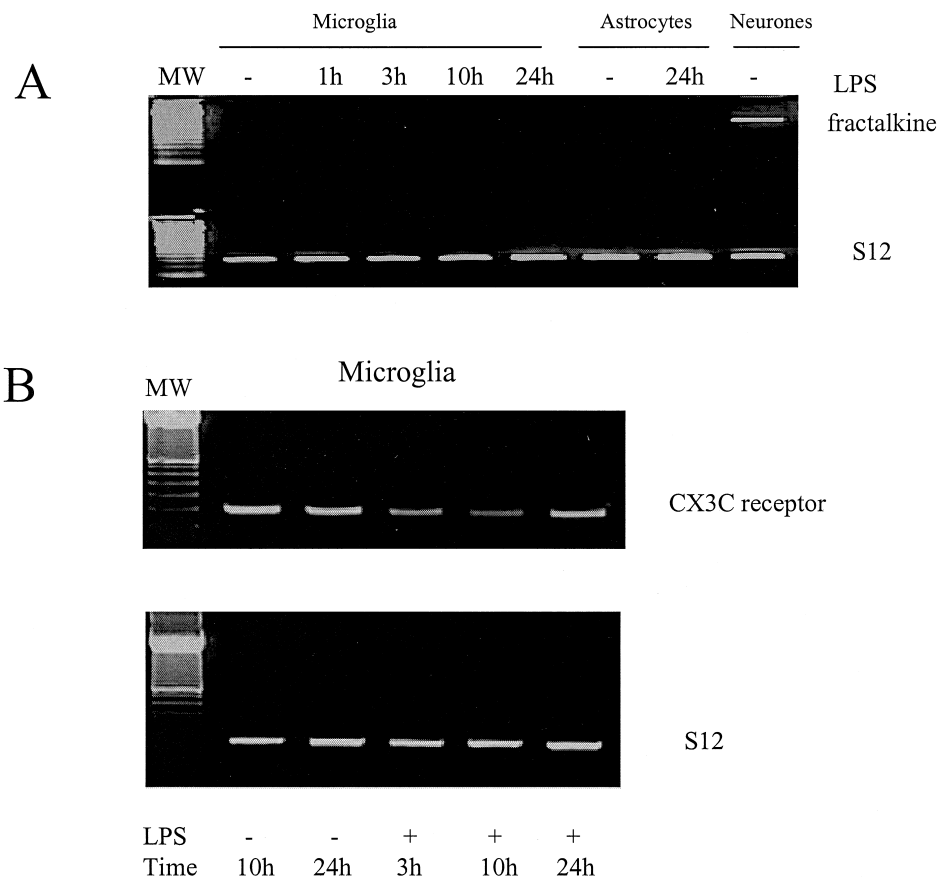

Fig. 1. (A) Expression of fractalkine measured in microglia, astrocytes and neurons. Strong expression of fractalkine mRNA is observed in neurons, whereas neither in presence nor in absence of lipopolysaccharide (100 ng/ml) expression is found in astrocytes and microglia. Data shown are typical experiments and have been repeated three times. (B) Suppression of fractalkine receptor mRNA expression in lipopolysaccharide-treated microglia. RT-PCR of fractalkine receptor mRNA shows no difference in expression of untreated microglia 10 h or 24 h in culture. 3 h and 10 h after exposure to lipopolysaccharide (100 ng/ml), a progressive down-regulation of fractalkine receptor mRNA is observed. 24 h after exposure to lipopolysaccharide (100 ng/ml) a recovery of fractalkine receptor mRNA expression is found. The lower bands show the internal standard cDNAs of S12.

rat fractalkine receptor (RBS11): Acc.# L09230; rat S12: Acc.#. M18547. Identity of PCR products was confirmed by cloning and subsequent sequencing.

2.2. Measurement of calcium transients

Microglia cells, cultured on glass coverslips, were loaded (30 min) with Fluo-3 AM in buffer containing: (in mM) NaCl 120, HEPES 5, KCl 6, $CaCl_2$ 2, MgCl 1, glucose 5, $NaHCO_3$ 22, Fluo-3 AM 0.005; pH 7.4; (37°C). Fluorescence was quantified at excitation and emission wavelengths of 495 and 510 nm, respectively, using an HR Deltaron 1700 camera (Fuji Photochemicals®) mounted on an inverted microscope. Peak amplitudes of calcium transients induced by fractalkine were expressed as percentage of the fluorescence induced by subsequent application of the calcium ionophore ionomycin (1 μM). In some experiments the cells were pretreated with thapsigargin (10^{-6} M; 20 min) or with ryanodine (10^{-6} M; 30 min) and washed before the experiment. In other 'calcium free' experiments calcium was omitted from the extracellular buffer and 1 mM EGTA was added. Statistical significance was estimated using the paired Student's *t*-test or the two-way analysis of variance (ANOVA) followed by Dunnets multiple comparison test. The significance level was set at $P < 0.05$. All data are presented as means ± S.E.M. (standard error of the mean).

2.3. Reagents

Compounds were obtained from the following sources: lipopolysaccharide: Pan Systems (Aidenbach, Germany). Fluo-3 acetylmethoxy ester (Fluo-3 AM): Molecular Probes (Oregon, USA). Ionomycin: Serva (Heidelberg, Germany). Dulbecco's modified Eagle's medium (DMEM): GIBCO-BRL (Basel, Switzerland). Fetal calf serum: Metalon (K-Wusterhausen, Germany). Taq polymerase, dNTPs: In-Vitek, (Berlin, Germany). Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), RNase inhibitor and Dithiotreitol: Gibco-BRL (Eggenstein, Germany). Recombinant soluble human fractalkine: R&D systems (Minneapolis, USA). Oligonucleotide primers: Birsner and Grob (Freiburg, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

3. Results

Using the reverse transcriptase (RT) PCR method, expression of fractalkine mRNA was found in enriched

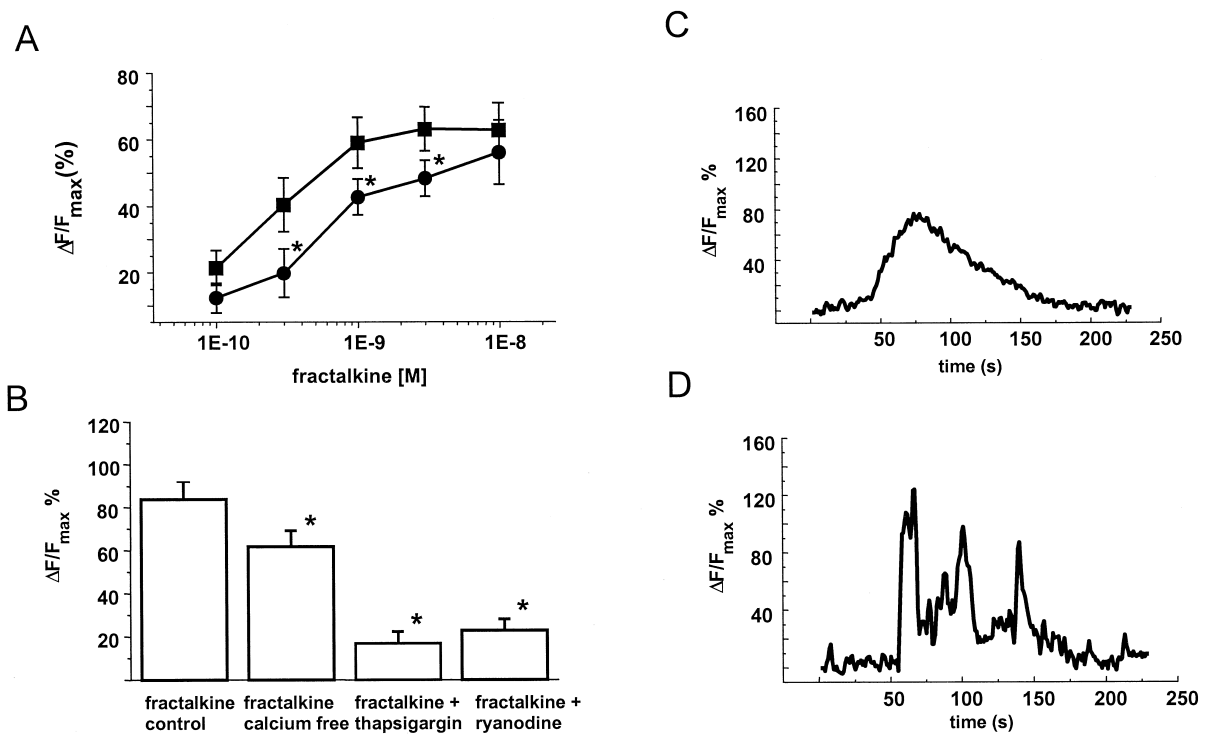

Fig. 2. (A) Concentration–response curve of the peak amplitude of the intracellular calcium response to fractalkine in non-treated (■) and lipopolysaccharide (100 ng/ml) treated (12 h; ●) rat microglia. The vertical axis represents the calcium response to fractalkine, the horizontal axis represents concentrations of fractalkine applied. The data represent mean values ± S.E.M. ($n = 15–34$). Asterisks indicate significant difference ($P < 0.05$). (B) Effect of fractalkine (3×10^{-9} M) on intracellular calcium release in presence and absence of extracellular calcium and after treatment with thapsigargin (10^{-6} M) or ryanodine (10^{-6} M). Asterisks indicate significant difference ($P < 0.05$; vs. the control fractalkine (3×10^{-9} M) experiment). Data represent mean values ± S.E.M. ($n = 7–11$). (C) Example of a calcium response to fractalkine (3×10^{-9} M) in single rat microglia cells. (D) Administration of high concentrations of fractalkine (10^{-8} M) induced characteristic oscillatory calcium transients. The vertical axis represents increases of calcium-mediated fluorescence expressed as $\Delta F/F_{max}$. The horizontal axis represents the time (s).

cortical neuronal cultures. By contrast, no expression of fractalkine mRNA was observed in primary astrocyte and microglia cultures (Fig. 1A). Fractalkine receptor mRNA was clearly expressed in cultured rat microglia. This expression did not change significantly within 10 h and 24 h after plating of cells. Incubation with lipopolysaccharide (100 ng/ml), however, suppressed expression of fractalkine receptor mRNA in a time-dependent manner (Fig. 1B). The strongest down regulation of fractalkine receptor mRNA was observed at 10 h of incubation with lipopolysaccharide (Fig. 1). After 24 h incubation with lipopolysaccharide, partial recovery of fractalkine receptor mRNA expression was observed (Fig. 1B).

Administration of fractalkine to primary cultured microglia induced a concentration-dependent (10^{-10}–10^{-8} M) increase of the intracellular calcium concentration (Fig. 2A). Pretreatment with lipopolysaccharide (12 h) induced a shift to the right of the concentration–response curve of the calcium response to fractalkine (Fig. 2A). This difference was significant ($P < 0.05$) at fractalkine concentrations of 3×10^{-10} and 3×10^{-9} M (Fig. 2A). Whereas at a low concentration (10^{-9} M) fractalkine induced a smooth calcium transient, at high concentrations (10^{-8} M) a pronounced, oscillatory response was observed (Fig. 2C,D). The response to fractalkine (10^{-8} M) was weakly, but significantly ($P < 0.05$) affected by omission of extracellular calcium but was strongly suppressed after pretreatment of microglia cells with the calcium store-depleting compound thapsigarigin (Fig. 2B). Furthermore, pretreatment with the calcium-induced calcium release inhibitor ryanodine strongly inhibited the response to fractalkine (Fig. 2B), suggesting that calcium-induced calcium release is involved in the effect of fractalkine.

4. Discussion

Recently, a new chemokine called fractalkine, which is abundantly expressed in brain tissue of humans and rodents, has been described. Fractalkine is mainly expressed in neurons, whereas its receptor is specifically expressed in microglial cells, thus suggesting a paracrine interaction between neurons and microglia (Nishiyori et al., 1998; Harrison et al., 1998). A possible function of this 'neuronal' chemokine is the establishment of a chemotactic gradient for leukocytes. This is supported by the fact that microglia perform fractalkine-induced chemotaxis (Harrison et al., 1998). It is currently not known whether the expression of the fractalkine receptor is regulated by inflammatory stimuli. We have addressed this question by investigating the effect of lipopolysaccharide on functional expression of the fractalkine receptor in primary rat microglia.

Our data show that fractalkine receptor mRNA expression is transiently suppressed in the cells during incubation with lipopolysaccharide. The concentration–response curve of the soluble form of fractalkine in microglia is shifted to the right 12 h after incubation with lipopolysaccharide. Bacterial endotoxin is often used to study inflammatory reactions and to induce the production and release of inflammatory mediators such as cytokines and chemokines in microglia both in vivo and in vitro (Gebicke-Härter et al., 1989; Buttini and Boddeke, 1995; Ulevitch and Tobias, 1995). Accordingly, a range of inflammatory mediators such as the cytokines interleukin-1β and tumor necrosis factor-α as well as chemokines such as macrophage inflammatory protein-1α, -1β and monocyte chemoattractant protein-1 are upregulated after treatment with lipopolysaccharide (Gourmala et al., 1997; Berczi, 1998). In view of the lipopolysaccharide-induced upregulation of these proinflammatory factors, the down-regulation of fractalkine receptor mRNA by lipopolysaccharide seems unexpected. Since under the culture conditions used fractalkine expression was observed neither in astrocytes nor in microglia the lipopolysaccharide-induced down-regulation of the functional expression of the fractalkine receptor cannot be explained by endogenous fractalkine production. Recently, upregulation of fractalkine receptor mRNA after facial motor nerve axotomy has been described (Harrison et al., 1998). The increased hybridization signal reported by these investigators presumably is due to an increased number of microglia accumulating in the lesioned area and thus is not at variance with an lipopolysaccharide-induced downregulation of fractalkine receptor mRNA expression, as observed in this study.

The small (8 kDa) soluble fractalkine fragment (10^{-10}–10^{-8} M) induced pronounced calcium transients. The calcium responses, particularly to high concentrations of soluble fractalkine were transient and oscillatory. To our knowledge, oscillatory calcium responses have not been observed for any other chemokine. Furthermore, the calcium response to fractalkine seems to be mediated primarily by calcium-induced calcium release.

Whereas, the soluble fractalkine fragment was shown to activate the receptor in recombinant systems (Imai et al., 1997; Harrison et al., 1998), in a recent study no calcium transients could be detected in rat microglia (Harrison et al., 1998). These investigators, however, performed their calcium imaging experiments on microglia cell suspensions. Methodological differences may thus account for the different results. Furthermore, chemotactic activity of microglia activated by the soluble fractalkine fragment has been described (Harrison et al., 1998), which suggests that the soluble fragment is capable of receptor activation.

The present data show down-regulation of fractalkine receptor mRNA expression by lipopolysaccharide, which is reflected by a decrease in the response to soluble fractalkine in microglia. Although the regulation of inflammatory factors by lipopolysaccharide in vitro essentially parallels that observed in vivo, further experiments are warranted to evaluate the regulation of the fractalkine receptor in inflamed brain tissue.

References

Baggiolini, M., 1998. Chemokines and leukocyte traffic. Nature 392, 565–568.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., Schall, T.J., 1997. A new class of membrane-bound chemokine with a CX_3C motif. Nature 385, 640–644.

Berczi, I., 1998. Neurohormonal host defense in endotoxin shock. Ann. N.Y. Acad. Sci. 840, 787–802.

Buttini, M., Boddeke, H.W.G.M., 1995. Peripheral lipopolysaccharide stimulation induces interleukin-1β messenger RNA in rat brain microglial cells. Neuroscience 65, 523–530.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Gebicke-Härter, P.J., Bauer, J., Schobert, A., Northoff, H., 1989. Lipopolysaccharide free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J. Neurosci. 9, 183–194.

Gourmala, N.G., Buttini, M., Limonta, S., Sauter, A., Boddeke, H.W.G.M., 1997. Differential and time-dependent expression of monocyte chemoattractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration. J. Neuroimmunol. 74, 35–44.

Harrison, J.K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R.K., Streit, W.J., Salafranca, M.N., Adhikari, S., Thompson, D.A., Botti, P., Bacon, K.B., Feng, L., 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX_3CR1-expressing microglia. Proc. Natl. Acad. Sci. U.S.A. 95, 10896–10901.

Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., Yoshie, O., 1997. Identification and molecular characterization of fractalkine receptor CX_3CR1, which mediates both leukocyte migration and adhesion. Cell 91, 521–530.

Jiang, Y., Salafranca, M.N., Adhikari, S., Xia, Y., Feng, L., Sonntag, M.K., deFiebre, C.M., Pennell, N.A., Streit, W.J., Harrison, J.K., 1998. Chemokine receptor expression in cultured glia and rat experimental allergic encephalomyelitis. J. Neuroimmunol. 86, 1–12.

Nishiyori, A., Minami, M., Ohtani, Y., Takami, S., Yamamoto, J., Kawaguchi, N., Kume, T., Akaike, A., Satoh, M., 1998. Localization of fractalkine and CX_3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia?. FEBS Letters 429, 167–172.

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J.C., Gearing, D., 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature 387, 611–617.

Spleiss, O., Gourmala, N., Boddeke, H.W.G.M., Sauter, A., Fiebich, B.L., Berger, M., Gebicke-Haerter, P.J., 1998. Cloning of rat HIV-1-chemokine co-receptor CKR5 from microglia and upregulation of its mRNA in ischemic and endotoxinemic rat brain. J. Neurosci. Res. 53, 16–28.

Ulevitch, R.J., Tobias, P.S., 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Ann. Rev. Immunol. 13, 437–457.