Role of cholecystokinin-8 in nerve growth factor and nerve growth factor mRNA expression in carrageenan-induced joint inflammation in adult rats

L. Manni1,2, T. Lundeberg2, P. Tirassa1 and L. Aloe1

1Institute of Neurobiology and Molecular Medicine, CNR, Viale Marx 15, 00137 Rome, Italy and 2Department of Physiology and Pharmacology, Karolinska Institute, S17177 Stockholm, Sweden

Abstract

Objectives. The aim of our study was to investigate the role of cholecystokinin-8 (CCK-8), which is able to induce the synthesis of nerve growth factor (NGF), in the joint inflammation of carrageenan-injected rats.

Methods. Adult rats were injected in the ankle joint with carrageenan, with or without CCK-8 or a CCK receptor antagonist (proglumide), and tissue swelling, NGF levels and NGF mRNA expression were assessed.

Results. Expression of NGF and NGF mRNA increased transiently after carrageenan injection. This effect was not altered by CCK-8 injection but was inhibited by the CCK receptor antagonist. The decrease in NGF level after treatment with the antagonist was concurrent with an increase in paw swelling.

Conclusions. The results demonstrate that, whereas CCK-8 has no anti-inflammatory action in carrageenan-injected animals, proglumide induces a worsening of inflammation and reduces the expression of both NGF and NGF mRNA in inflamed ankle joints. Our data point to a regulatory action of CCK-8 on NGF synthesis during acute synovitis and suggest a role for NGF in the healing phase of inflammation.

Key words: Arthritis, Carrageenan, Cholecystokinin-8, Nerve growth factor, Rats.

Nerve growth factor (NGF) is a neurotrophin that has a pivotal role in the regulation of the growth and differentiation of peripheral sensory and sympathetic neurons [1], in the modulation of neurotransmitter and neuropeptide synthesis and in the recovery of lesioned peripheral nerves [1, 2]. Alterations in serum and tissue NGF levels were recently observed in patients during the course of inflammatory and autoimmune diseases [3–5]. Similarly, data obtained from a number of different animal models, showing that inflammation is associated with a significant increase in basal NGF level [5], suggest that NGF might play a role in the inflammatory response and that NGF itself might promote or exacerbate inflammation [6–8]. However, the latter hypothesis is controversial, as it was observed that NGF can promote the repair of damaged tissue [9, 10] and exert anti-inflammatory action in humans and in animal models of inflammation [9, 11–13].

Studies carried out in our laboratory also indicate that joint inflammation, both in animal models and in humans, is characterized by increases in local and systemic NGF levels [14–16]. The up-regulation of NGF is associated with increased expression of pro-inflammatory cytokines, such as interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α), in the inflamed synovium [14, 17]. We also observed that injection of NGF into the joint did not induce inflammation [16], suggesting that the action of NGF at the site of inflammation might be related more to reparative events than to the progression of inflammation. To gain further insight into the role played by NGF during inflammatory joint disease, we asked whether and how the modulation of NGF synthesis might affect the course of inflammation. We used a biological mediator that is able to enhance endogenous NGF synthesis.

We demonstrated recently that the neuropeptide cholecystokinin-8 (CCK-8) is able to induce over-expression of NGF and to promote functional and structural recovery of chemically and/or surgically damaged nerve cells [18–21]. In the present study, the effects of daily treatment with CCK-8 on NGF...
expression and the progression of joint inflammation were analysed in a model of carrageenan-induced arthritis [22]. As the CCK-induced increase in NGF is blocked by the administration of CCK receptor antagonists [21], we also studied the effects of proglumide, a compound that antagonizes the binding of CCK to both the CCK<sub>A</sub> and the CCK<sub>B</sub> receptor [23].

Materials and methods

Animal care

Adult female Sprague–Dawley rats (mean weight ± S.D. 203 ± 18 g) were purchased from Charles River Italy (Calco, Italy). The animals were housed in plastic holding cages under standard laboratory conditions (temperature 21 ± 1°C, relative humidity 60 ± 10%, lights on from 09:30 a.m. to 09:30 p.m.). Animal care and procedures conformed with the intramural committee and institutional guidelines in accordance with national and international laws (EEC council directive 86/609, OJ L 358, 1, 12 December 1987).

Treatments and experimental procedures

Acute arthritis was induced in adult female rats (n = 25) by injecting into the ankle joints, after mild ether anaesthesia, 100 μl of 2 mg/ml λ-carrageenan (Sigma, St Louis, MO, USA) dissolved in 0.9% sodium chloride solution (saline). A second group of rats (n = 5) were injected with 100 μl of saline and served as controls. The severity of arthritis was evaluated by measuring paw volume (see below). Groups of five carrageenan-treated rats were killed with an overdose of sodium pentobarbital 3, 7, 14, 21 and 28 days after the induction of arthritis. The joint was removed and frozen immediately, and stored frozen until used for determination of NGF or NGF mRNA.

To evaluate the effect of CCK-8 on NGF synthesis during joint inflammation, two groups of 20 rats were injected into the ankle joints with carrageenan and saline respectively. Six hours after the induction of arthritis, the rats were further divided into four groups and given a daily intraperitoneal (i.p.) injection of saline solution or 8 nmol/kg of CCK-8 (Peninsula Laboratories Inc., San Carlos, CA, USA) dissolved in saline solution. Under these conditions, recovery was greater than 90%.

To further investigate the effect of CCK-8 on NGF synthesis, a group of 20 rats were injected into the joint with carrageenan as described above, and a second group of 20 rats were injected with saline solution. Six hours after the induction of arthritis, rats were further divided into four groups and daily injected i.p. with 1 ml of a 2% solution of the non-selective CCK receptor antagonist proglumide (Sigma) [23] dissolved in 60% saline and 40% DMSO (dimethyl sulphoxide; vehicle solution), or with vehicle solution only. The four groups were as follows: (i) saline-treated, injected with vehicle (n = 10; vehicle controls); (ii) saline-treated, injected with proglumide (n = 10; proglumide controls); (iii) carrageenan-injected and treated with vehicle (n = 10; untreated arthritis); (iv) carrageenan-injected and treated with proglumide (n = 10; proglumide-treated arthritis). Four days after the induction of inflammation, the severity of arthritis was evaluated by measuring paw volume (see below), then the animals were killed and tissue was removed and frozen until used for determination of NGF or NGF mRNA.

Assessment of paw volume

Hind-paw volume was measured with an electronic plethysmometer (Model 7150; Ugo Basile, Modena, Italy) and used as an index of the severity of the swelling resulting from arthritis [24]. In the time-course study, the result for each time-point was calculated as a percentage increase with respect to the baseline value (time-point zero) and repeated-measures analysis of variance (ANOVA) was used to evaluate significant differences between groups of treatment and/or time points (see Statistical evaluation, below). In all other experiments, paw volume (expressed in ml) was used for comparison among the experimental groups.

NGF determination by ELISA

Mice were killed and the joint was removed, frozen quickly at –70°C and stored frozen until the NGF assay was performed. The levels of NGF were measured using a monoclonal mouse anti-NGF antibody (clone 27/21; Boehringer Mannheim, Mannheim, Germany) coated on polystyrene 96-well immunoplates for capturing of NGF from samples and standard curve. Anti-β-NGF-galactosidase (clone 27/21; Boehringer Mannheim) was used for the colorimetric detection [25, 26]. The recovery of NGF during the assay procedure was estimated by adding a known amount of highly purified NGF to the samples or to the homogenization buffer as an internal control. The yield of exogenous NGF was calculated by subtracting the amount of endogenous NGF from the total of endogenous and exogenous NGF. Under these conditions, recovery was greater than 90%.

RT–PCR ELISA

The levels of NGF mRNA in the joints were evaluated using the reverse transcriptase–polymerase chain reaction (RT–PCR) enzyme-linked immunosorbent (ELISA) protocol described by Tirassa et al. [27]. Briefly, total RNA was extracted from the joint by the method of Chomczynski and Sacchi [28] as modified in the TRIzol Kit (Invitrogen Italia SRL, San Giuliano Milanese, Italy). Complementary DNA was synthesized from 1 μg of total RNA using 200 U of M–MLV reverse transcriptase (Promega Italia, Milan, Italy) in a total
reaction volume of 20 µl. The NGF and Gapdh (glyceraldehyde-3-phosphate dehydrogenase) genes were co-amplified in a single-tube PCR reaction (30 cycles: 1 min at 95°C, 1 min at 55°C, 2 min at 72°C) using 5′-biotinylated specific primers to generate biotinylated PCR products detectable by digoxigenin-labeled probes in an immunoenzymatic assay. The amount of amplified products was measured as optical density at 450/690 nm (OD$_{450/690}$) using a Dynatech ELISA Reader 5000 (PBI International Milan, Italy). The GAPDH OD$_{450/690}$ level was used to normalize for the relative differences in sample size, integrity of the individual RNA and variation in the efficiency of reverse transcription. For details of methods and the primer/probe sequences, see Tirassa et al. [27].

Statistical analysis
The time course of NGF and NGF mRNA expression in the ankle joints was evaluated by ANOVA using the StatView package for Macintosh (Abacus Concepts, Berkeley, CA, USA) and data are expressed as mean ± s.e.m. The time-course effects of carrageenan administration on paw volume were evaluated with the repeated measures (six tests) and treatments (saline, carrageenan) as factors. Data are expressed as percentage increase with respect to the baseline value (time-point zero). All other data were subjected to ANOVA, with arthritis and CCK-8 or proglumide treatment as variables. Data are expressed as mean ± s.e.m. Post hoc comparisons within logical sets of means were performed using Tukey’s test (the use of which is permissible or even recommended in the absence of significant main or interaction effects in ANOVA in order to minimize frequency errors of both type I and II) according to Wilcox [29].

Results

Time-course study
In the first experiment, we evaluated the time course of the expression of NGF and its mRNA in inflamed tissue and the time course of paw oedema after the induction of acute inflammation. As shown in Fig. 1A, injection of adult rats with carrageenan induced a transient increase in paw volume. ANOVA revealed a main effect of arthritis induction [$F(1,5) = 21.18; P < 0.0001$] and an interaction between arthritis induction and repeated measures [$F(5,50) = 13.02; P < 0.0001$]. Post hoc comparisons showed that the increase in paw volume peaked significantly at day 3 after carrageenan administration and then returned to the baseline level by day 7.

The amount of NGF in the ankle joint (Fig. 1B) increased during the first 2 weeks after the induction of arthritis, reaching its maximum value 7 days after injection of carrageenan. As shown in Fig. 1B and C, the increase in NGF protein was concurrent with the increase in NGF mRNA; both NGF protein and mRNA thus increased between day 3 and day 14 after the induction of arthritis before returning to its normal level.

Fig. 1. Time-course of (A) paw volume, (B) NGF concentration and (C) NGF mRNA expression in the ankle joint of rats treated with carrageenan. Values are mean ± s.e.m. *$P < 0.05$ vs baseline value in post hoc analysis.

Effect of treatment with CCK-8
On the basis of the results of the time-course study, normal and arthritic rats were treated with CCK-8 for 4 days after carrageenan administration. The effects of the CCK-8 treatment on paw volume and NGF expression in the joint were analysed. As shown in Fig. 2, the effect of carrageenan injection on paw volume was confirmed, whereas no effect was detected for the treatment with CCK-8. Indeed, ANOVA and post hoc analysis did not reveal any significant alteration of paw volume in either controls or arthritic rats receiving CCK-8 injections. Moreover, levels of NGF and NGF mRNA (Fig. 2B and C) increased in the joint 4 days
after injection with carrageenan. The treatment of control rats with CCK-8 did not exert any significant effect on NGF and NGF mRNA, and only NGF mRNA expression was affected by treatment with CCK-8. Carrageenan treatment also led to an increase in NGF concentration that was not affected by CCK-8 injection. NGF mRNA (C) was also increased by carrageenan administration and was down-regulated by CCK-8. Indeed, post hoc analysis (Fig. 2C) revealed that CCK-8 decreased NGF mRNA expression in arthritic rats, even though expression remained significantly higher than in the saline control group ($P = 0.0164$ for the interaction effect in the ANOVA; $P < 0.05$ in the post hoc analysis).

**Effect of treatment with proglumide**

As in the previous experiment, joint swelling and NGF levels were evaluated 4 days after carrageenan and/or proglumide treatment. ANOVA and post hoc analysis showed that paw volume increased after carrageenan treatment ($P < 0.05$ in the post hoc analysis when the vehicle control and untreated arthritis groups were compared) and that injection with proglumide significantly exacerbated the effects of carrageenan ($P < 0.05$ when the untreated arthritis and proglumide-treated arthritis groups were compared; see Fig. 3A).

As shown in Fig. 3B, the enhanced swelling was associated with a reduction in NGF. NGF was increased 4 days after carrageenan treatment, and injections of proglumide into arthritic animals counteracted the effect of carrageenan by lowering NGF to the baseline
level. Both the induction of arthritis and proglumide treatment also affected NGF mRNA expression. Tukey’s test confirmed the increased expression of NGF mRNA in the untreated arthritis group 4 days after injection of carrageenan and the counteraction by proglumide of the effect of carrageenan treatment ($P < 0.05$ when the untreated arthritis and proglumide-treated arthritis groups were compared) (Fig. 3C).

Discussion

A variety of studies show that the inflammatory response, both in animal models and in humans, is characterized by increased expression of NGF [5, 6, 8] and that joint inflammation results in local over-expression of NGF [3, 17, 30] and of NGF receptors [30]. The role of neurotrophins in this type of inflammatory response is, however, still not clear, as the presence of elevated levels of NGF in arthritic joints has been described as both pro- and anti-inflammatory [3, 17, 31]. The present study aimed to provide a better characterization of the role of NGF in joint inflammation by studying the progression of carrageenan-induced joint inflammation and the effect of endogenous stimulation on NGF synthesis in inflamed joints, which were obtained by injecting CCK-8 or the CCK receptor antagonist proglumide.

Our studies showed that joint inflammation induces transient increases in NGF and NGF mRNA in the joint. Although our work was not intended to identify the cell type(s) responsible for NGF synthesis, our observation of increased NGF mRNA indicated that the increased level of NGF in inflamed joints is due to local production.

The evidence that the peak expression of NGF during carrageenan-induced inflammation corresponds to the reduction in joint swelling (Fig. 1) suggests that the up-regulation of NGF might be implicated in the healing phase of inflammation. This is in line with previous studies demonstrating that NGF plays a crucial role in promoting tissue healing in both animal models and in humans [9, 10] and that the injection of purified NGF into the knee joint did not induce local inflammation [16]. It has been reported recently that blocking the activity of cyclooxygenase (COX) metabolites by the administration of non-steroidal anti-inflammatory drugs is able to reduce paw swelling without affecting the local overexpression of NGF induced by carrageenan [32]. These findings point to NGF as only one of the players in the long-lasting sensitization of primary afferent neurones during acute inflammation [32]. Nevertheless, a role for NGF as an anti-inflammatory agent, through its modulatory action on the peripheral nervous system, was recently envisaged in the use of different models of inflammatory disease [12, 13, 33]. Overall, these pieces of evidence support the hypothesis that NGF, under certain conditions, also exerts an anti-inflammatory effect on inflamed joints.

Because CCK-8 promotes NGF synthesis [18–21], in order to test the hypothesis of an anti-inflammatory action of endogenous NGF we treated normal and carrageenan-injected rats with CCK-8 or with the CCK receptor antagonist proglumide [23]. Indeed, the regulatory effect of CCK-8 on NGF was demonstrated to be functionally relevant, because the CCK-induced NGF overexpression was able to trigger and/or accelerate the healing process in chemically induced neuropathies [18, 19].

Unlike what was previously observed in animal models of central and peripheral nerve lesions [18–21], administration of CCK-8 during joint inflammation seems to have no effect on NGF synthesis. Why the administration of CCK-8 does not induce the up-regulation of NGF in inflamed joints is not known. No evidence has yet been found about the presence of CCK-8 receptors on NGF-producing cells in the joint. However, it has been reported that CCK-8, through the activation of CCK receptors expressed by monocytes/macrophages, may influence the production of pro-inflammatory cytokines [34]. Thus, the possibility exists that the action of CCK-8 on immune cells infiltrating the inflamed synovia might result in an effect on neurotrophin expression by these NGF-producing cells [35]. Our observation that the administration of proglumide, a non-selective CCK receptor antagonist [23], causes a decrease in the local expression of NGF suggests a regulatory/stimulatory action of endogenous CCK-8 on NGF synthesis. The identity of the cells responsible for NGF production and the mechanism(s) of the regulatory action of CCK on NGF expression during carrageenan-induced joint inflammation require further investigation.

In a recent report, the expression of both NGF and its receptors, p75 and TrkA, have been reported in inflamed joints of adjuvant-induced arthritic rats [30], and our preliminary data (L. Manni et al., unpublished observation) also revealed increased expression of TrkA in carrageenan-treated rat ankle joints. The concomitant expression of NGF and its receptors in joint tissues suggests that the neurotrophin could act, at least in part, by modulating the inflammatory process via an autocrine/paracrine mechanism [30]. However, the fact that proglumide not only decreases NGF expression into the inflamed synovia might result in an effect on neurotrophin expression by these NGF-producing cells [35].

Acknowledgements

The authors thank Professor L. Calzà and Dr L. Giardino for providing us with the benefit of their experience in the measurement of paw volume with the electronic plethysmometer. This work was supported by Progetto Finalizzato ‘Biotech 5’ Subproject 3 CNR to L.A.
References