EFFECTS OF CHOLECYSTOKININ-8 IN PERIPHERAL NEUROPATHIES: A NERVE GROWTH FACTOR MEDIATED ACTION?

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CHOLECYSTOKININ-8

Cholecystokinin-8 (CCK-8) is a member of a gastrointestinal peptide family – which includes gastrin, cionin and cereulin – that was first identified and characterized in the gastrointestinal tract as a hormone with a major role in the regulation of gut motility, pancreatic secretion, and gallbladder contraction (18). Today it is known that CCK-8 is widely distributed in the nervous system and acts as a neuromodulator and a neurotransmitter controlling various physiological events, such as the processing of sensory information both in the spinal cord and at the primary afferent level. Moreover, in a recent report it was showed that the axotomy-induced enhancement of CCK expression by dorsal root ganglia (DRG) neurons was concurrent with the failure of nerve growth factor (NGF) supply by denervated target, and that NGF administration restored the normal expression of CCK in injured animals (50). Based on the above considerations and the observed capacity of CCK-8 to attenuate the effect of neuronal dysfunction following brain lesion in a NGF-dependent manner (46, 47), we performed experiments to verify if the "neuroprotective" action of CCK-8 could also be manifested in the peripheral nervous system (PNS).

PERIPHERAL NEUROPATHIES

The term "peripheral neuropathy" (PN) is used to encompass many distinct syndromes, which differ according to the etiology and to which neuronal population is actually affected. PN in humans may be induced by surgery or injuries or else may develop as a side effect after exposure to neurotoxic compounds, such as anti-neoplastic drugs, or as a disease-related syndrome (13). PN caused in patients by anti-neoplastic drugs is a serious side effect in the therapeutical utilisation of these compounds (13). PN is also one of the common complications in patients with diabetes mellitus, causing impairment of sensory neurones and resulting in impaired wound healing (52). A deficit of NGF production by innervated tissue has been reported in diabetic patients (27, 48), suggesting that the lack of target-derived trophic supply to innervating neu-

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rons could be a major pathogenetic factor in the development of diabetic neuropathy. For this reason and because of its role on survival, differentiation and regulation of neurotransmitter/neuropeptides synthesis in PNS neurons (37, 50), NGF has been identified as one of the most suitable therapeutic targets for the clinical treatment of PN (10).

NGF is a neurokine discovered about 50 years ago for its properties of stimulating growth and differentiation of PNS neurons (33). Since then a consistent number of studies have shown that NGF displays a wider range of action on a variety of cells within and outside the nervous system. Studies published in recent years have shown that NGF might be involved in a variety of neurological disorders, including peripheral neuropathies (41) and most probably in inflammatory (3) and autoimmune diseases (4). Clinical use of NGF for human brain disorders, peripheral neuropathies and cutaneous ulcers has been reported (10, 14, 39, 49). Despite the promising results so far obtained for cutaneous lesions, one key issue limiting the therapeutical utilization of NGF is the difficulty in identifying suitable agonist and/or regulatory molecules for NGF and NGF receptors. One major aim of our recent experimental work was to identify molecules involved in this process.

Recent studies on the utilisation of NGF in the treatment of human peripheral neuropathies have attracted great attention (11, 12, 41). Indeed in the past years clinical trials have evaluated the therapeutic impact of NGF treatment in patients with peripheral neuropathies. In accordance with the experimental data, an improvement of the functions of small-fiber sensory peripheral neurons was noted in diabetic patients with symptomatic neuropathy receiving recombinant human NGF (11, 12). Because of the reduced neuropathic symptoms and the fact that the NGF treatment is well tolerated by both healthy subjects and patients, the clinical trials have reached phase III. The results of a study conducted in the United States from 1997 through to 1999 showed that the treatment with recombinant NGF failed to produce beneficial effects on diabetic polineuropathy (12). One of the main reasons of this failure is that, although the patients show a significant improvement in the global symptoms, this is associated with the development of a pain syndrome manifested as intense pain in the legs and feet. It is interesting to note that the adverse effects are highly dependent on the dosage and the duration of the treatment. Indeed, dose-dependent NGF effect on pain was observed after intravenous or subcutaneous injection with the factor (16, 40) and in patients affected by peripheral neuropathies (11, 12). Many studies have been addressed to discover strategies to overcome these problems including the use of modified cell clones producing NGF (22), the conjugation with proteins which can increase the NGF diffusion rate or its half-life (25) and the discovery of substances stimulating endogenous NGF production (41, 43).

ANIMAL MODELS OF SENSORY AND SYMPATHETIC NEUROPATHIES

Several animal models of PN have been developed and used in preclinical studies. Among them, the streptozotocin-induced diabetes (17), and the neurotoxic-

induced selective neuropathy (28, 31) have probably revealed as the most useful to perform studies on neuroprotection and nerve repair mechanism(s) during PN.

Sensory Neuropathy: Sensory neuropathy can be established by treating adult rodents with the sensory neurotoxic compound capsaicin (28). Capsaicin is the most pungent ingredient of the red pepper, having a selective and higly specific neurotoxic action on type-C unmyelinated sensory fibres. Systemic injection of capsaicin in adult animals induces a loss of capsaicin-sensitive nerve fibres with long-lasting block of the sensitivity. This model has been extensively used in the past to characterize the role of NGF on survival and phenotypic maintenance of developing and adult sensory neurons (42, 51) and to identify a possible therapeutic role of NGF in the treatment of human sensory neuropathies.

Sympathetic Neuropathy:One animal model of peripheral sympathectomy is achieved by injecting adult animals with 6-hydroxydopamine (6-OHDA) (31). 6-OHDA is a sympatholitic drug that causes a selective sympathetic nervous system lesion (31). Uptake of 6-OHDA into noradrenergic nerve terminals induces a toxic reaction by production of free radicals and loss of membrane integrity. Treatment of adult animals with 6-OHDA causes a reversible sympathetic lesion with loss of terminals and long-term depletion of tissue catecholamines. Numerous studies, both in animal models and in humans, have demonstrated that sympathetic neurones are highly dependent on NGF not only during development, but also in adult life (23, 24, 30). Peripheral sympathectomy of laboratory animals is a useful experimental model for examining the action of biologically active compounds on the neuronal plasticity and the functional relationship between nerve cells and innervated organs (30). Specifically, the use of chemical sympathectomy has helped to characterise the role of neurotrophic factors in structural, biochemical and functional recovery of peripheral injured neuritis and has stimulated clinical interest in these molecules (21).

CCK-8 AND PERIPHERAL NEUROPATHIES

Studies performed in our laboratories have indicated that the gastric peptide CCK-8 – which has a NGF-mediated neuroprotective action on CNS neurons when injected intraperitoneally – may be one of the endogenous molecules with potential clinical and pharmaceutical relevance (46, 47).

In the first study (35) we demonstrated that daily injections with low amounts of CCK-8 were able to functionally recover the sensory impairment generated by injection of the neurotoxin, capsaicin. Rescue from sensory impairment was concurrent with an up-regulation of NGF expression in the target tissue – namely the hind-paw skin. Moreover, the skin content of sensory neuropeptide substance P (SP) and calcitonin gene-related peptide (CGRP) – both involved in the primary mediation of pain sensations (44, 49) and also known to be regulated by NGF (37, 50) – were increased by CCK-8 treatment. Thus using an animal model of sensory PN, these studies demonstrate for the first time that a peripheral neuropeptide may influence NGF synthesis and/or expression, and sensory neuropeptide levels in peripheral tissues. These findings suggest that – although the effects of a prolonged treatment

with CCK-8 remain to be elucidated – a systemic treatment with low doses of CCK-8 may represent a potentially useful strategy for promoting the recovery of impaired PNS function.

Attempting to extend these observations, we applied the same experimental paradigm to a model of sympathetic neuropathy induced by 6-OHDA injection (31). The study revealed that, after daily treatment with CCK-8, the sympathetic innervation of the iris was partially restored and NGF levels were up-regulated in the eyes of the 6-OHDA treated animals. Moreover, after 6-OHDA, NGF and NGFmRNA were found to be increased by the CCK-8 treatment in another dense sympathetically-innervated organ, the heart. Although no evidences supporting the direct action of CCK-8 on NGF expression in PNS are available, our overall results suggest that the reparative effect of CCK-8 on damaged PNS occurs via the stimulation of NGF synthesis. Consistent with this hypothesis is the evidence that the expression of NPY – a neuropeptide regulated by NGF (2, 38) – in the heart and eyes of sympathectomized animals is affected by CCK-8 administration.

Although further studies may be necessary to understand the mechanism through which CCK-8 influences the recovery of sympathetic neurons, the fact that i.p. injection with CCK-8 stimulates NGF synthesis in periphery prospects the possibility that CCK-8 may be useful for a therapeutic approach to diseases associated with sympathetic deficit. It has recently been reported that NGF exerts a protective action against postischemic dysfunction of coronary sympathetic innervation in dogs (1) while autonomic failure in patients affected by longstanding dizziness is associated with reduction of peripheral NGF synthesis (9). Our findings, showing that CCK-8 significantly influences endogenous NGF synthesis suggest the hypothesis of a potential utilization of CCK-8 to overcome or reduce NGF-related peripheral autonomic dysfunction.

MODULATION OF NGF DURING INFLAMMATORY ARTHRITIS

A variety of studies have shown that NGF is involved in inflammatory response (3, 7, 20). Several experimentally-induced autoimmune and inflammatory disorders are characterized by a transient increase of NGF (4). The functional role of NGF in these disorders has not yet been established, though we and other researchers have provided evidence of an anti-inflammatory action of NGF in animal models of joint inflammation (5-7, 36). Indeed, it was shown that NGF exerts anti-inflammatory action on human corneal inflammation (32).To further study the role of NGF in inflammation, we have used CCK-8 on an animal model of inflammatory arthritis. The rationale for this experimental approach was based on the assumption that CCK-8 would increase endogenous NGF, thus influencing the course of inflammation (36).

Our results indicated that the injection of adult rats with carrageenan – a pro-inflammatory compound used to induce experimental acute inflammation (26) – induced a rapid and transient increase in paw volume that peaks at day three after carrageenan administration and then returns to baseline levels by day seven. The
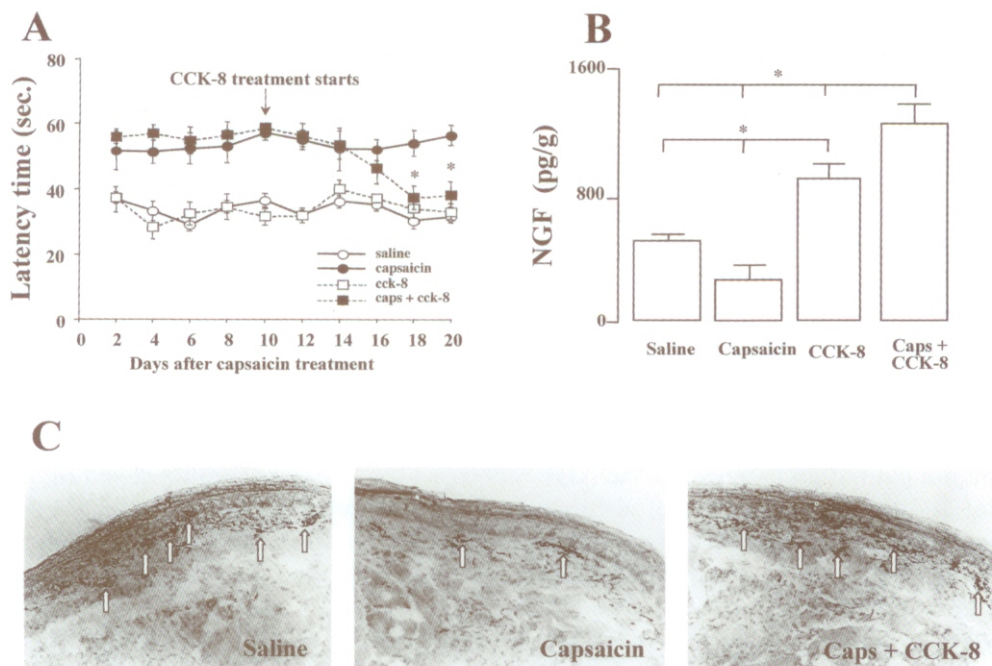
Fig. 1. - **A:** *Hot-plate response of adult mice treated with capsaicin.*
The latency time of the response to noxious heal in capsaicin-treated mice remains higher than in the controls for the entire observation period, while the treatment with CCK-8 induces a decrease of the response-latency time in capsaicin-treated mice. $^{*}p < 0.05$ when capsaicin and CAPS+CCK-8 groups are compared.
B: *Treatment for ten days with physiological amounts of CCK-8 increases NGF levels in normal mice and further enhances the neurotrophin expression in capsaicin-lesioned mice.* Data are plotted as mean ± SEM. $^{*}p < 0.05$.
C: *In situ hybridisation shows that NGF mRNA (withe arrows) is normally expressed in the dermal layer of the skin (saline).* The decreased expression of NGF mRNA observed after treatment with capsaicin (*Capsaicin*) was completely reversed by treatment with CCK-8 (*Caps+CCK-8*).

amount of both NGF and NGF-mRNA in the ankle joint increased between day three and day fourteen after the induction of arthritis before returning to its normal level, reaching its maximum value seven days post-carrageenan injection (36).

Unlike what has been previously observed in animal models of central and peripheral nerve lesions (34, 35, 46, 47), exogenous CCK-8 administration during joint inflammation does not have effect on both inflammatory edema and NGF synthesis. Why CCK-8 administration does not induce up-regulation of NGF in inflamed joints is actually 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 (19). Thus, the possibility exists that the action of CCK-8 on immune cells infiltrating the inflamed synovia, might possibly result in an effect on neurotrophin expression by these NGF-producing cells (15).

To further investigate this hypothesis and the role of CCK on NGF expression during experimental arthritis, we performed experiments evaluating joint swelling and NGF levels after carrageenan and/or proglumide – a non-selective CCK-receptor antagonist (29) – treatment. Our results revealed that injection with proglumide significantly exacerbated carrageenan effects on paw edema and that the enhanced swelling was associated with a reduction of NGF. Indeed NGF increased four days after carrageenan and proglumide injections into arthritic animals and counteracted the carrageenan effect by lowering NGF and NGFmRNA to baseline levels. The observation that administration of proglumide, causes a decrease of local expression of NGF, further suggests a regulatory/stimulatory action of CCK-8 on NGF synthesis. The identification of the cells responsible for NGF production and the mechanism(s) of CCK regulatory action on NGF expression during carrageenan-induced joint inflammation require further investigation. Moreover the immunohistochemical analysis carried out on joint sections revealed that carrageenan administration caused an increase of the NGF receptor (TrkA) expression localised in the joint and that the treatment of arthritic animals with proglumide counteracted the effect of carrageenan, reducing the TrkA immunostaining (Manni *et al.*, unpublished). The concomitant enhanced expression of NGF and TrkA in joint tissues suggests that the neurotrophin could act, at least in part, by modulating the inflammatory process via an autocrine/paracrine mechanism. The fact that proglumide, not only decreases NGF and TrkA expression into the joint of arthritic rats, but also enhances the inflammatory condition, further suggests that NGF expression and/or utilisation might be associated with healing rather than trigger or exacerbate inflammation.

POTENTIAL PHARMACOLOGICAL RELEVANCE

It has been reported that NGF can be clinically useful in promoting healing in human peripheral neuropathies such as those induced by diabetes (11), leprosy (8) or AIDS (45). Unfortunately, exogenous administration of NGF can cause transient peripheral hyperalgesia rendering the clinical utilization of this molecule unpleasant for the patients (11). Our studies provide evidence that CCK-8 can influence the endogenous production of NGF. These observations indicate that systemic treatment with low doses of CCK-8 may represent a potentially useful therapeutic strategy for the treatment of peripheral neuropathies, by promoting the recovery of NGF-responsive nerve cells. Indeed, our works show that CCK-8 is able to induce NGF synthesis in the absence of side effects, such as hyperalgesia, observed with exogenous administration of NGF (35). Although many aspects of the neuroprotecive action exerted by CCK-8 need to be elucidated, our results lead to the hypothesis that it might be possibile to take advantage of the functional interaction between NGF and CCK-8 to develop more selective and safe pharmacological agents.

SUMMARY

Cholecystokinin-8 (CCK-8) is a member of a gastrointestinal peptide family – which includes gastrin, cionin and cereulin – that was first identified and characterized in the gastrointestinal tract as a hormone with a major role in the regulation of gut motility, pancreatic secretion, and gallbladder contraction (18). In the present investigation we report that administration of CCK-8 is able to stimulate peripheral sensory and sympathetic nerve fibers previously axotomized by neurotoxic compounds such as capsaicin and 6-OHDA respectively. Though the mechanism is not yet been fully elucidated, biochemical and molecular studies suggest that the effect of CCK-8 is mediated by upregulation of NGF, a molecule wich plays a crucial role in peripheral nervous system regeneration.

The potential pharmacological relevance of these findings is discussed.

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