Role of Thymulin or Its Analogue as a New Analgesic Molecule

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ABSTRACT: **The thymic peptide thymulin is known for its immunomodulatory role. However, several recent reports have indicated that thymulin is capable of interacting directly and/or indirectly with the nervous system. One of the first lines of evidence of this interaction was obtained in a series of experiments showing the hyperalgesic actions of this peptide. We demonstrated that, at low doses (ng), local (intraplantar) or systemic (intraperitoneal) injections of thymulin resulted in hyperalgesia with an increase in proinflammatory mediators, and that this peptide could act directly on the afferent nerve terminals through prostaglandin-E2 (PGE2)-dependent mechanisms, thus forming a neuroimmune loop involving capsaicin-sensitive primary afferent fibers. In further experiments, systemic injections of relatively high doses (1–25 μg) of thymulin or of an analogue peptide (PAT) deprived of hyperalgesic effect, have been shown to reduce the inflammatory pain and the upregulated levels of cytokines induced by endotoxin (ET) injection. In addition, PAT treatment appeared to alleviate the sickness behavior (motor behavior and fever) induced by systemic inflammation. These effects could be attributed, at least partly, to the downregulation of proinflammatory mediators. Furthermore, when compared with the effects of other anti-inflammatory drugs, PAT exerted equal or even stronger analgesic effects, and at much lower concentrations. Subsequent experiments were designed to examine the effects of intracerebroventricular (i.c.v.) injections of thymulin on cerebral inflammation induced by i.c.v. injection of ET. Pretreatment with thymulin reduced, in a dose-dependant manner, the ET-induced hyperalgesia, and exerted differential effects on the upregulated levels of cytokines in different areas of the brain, suggesting a neuroprotective role for thymulin in the central nervous system (CNS). Preliminary results demonstrate that thymulin inhibits in the hippocampus the ET-induced nuclear activation of NF-κB, the transcription factor required for the expression of proinflammatory cytokines genes. Although the**

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mechanism of action of these molecules is not totally elucidated, our results indicate a possible therapeutic use of thymulin or PAT as analgesic and anti-inflammatory drugs.

KEYWORDS: **thymulin; PAT (peptide analogue of thymulin); hyperalgesia; analgesics; inflammation; cytokines; thymus**

INTRODUCTION

Thymulin, a highly conserved nonapeptide synthesized by two discrete populations of thymic epithelial cells, has been characterized mainly for its role in the immune system.[1] It has been shown to have two major actions on T cells and their immature precursors; first, induction of differentiation markers and second, enhancement of various T and NK cell activities.[2] Thymulin requires the presence of zinc to express its biological activity.[3]

More recent evidence points to an important role for thymulin as a signaling molecule for interaction between the immune, endocrine, and the nervous system.[4] Immunohistochemical techniques have shown the presence of this peptide in astrocytic glial cells in the brain and skin basal keratinocytes.[5] Several studies have described a bidirectional communication between the thymic epithelium and the hypothalamus-pituitary axis.[6] For example, thymulin has been shown to be released into the blood with a circadian rhythm coinciding with the activity of the hypothalamus-pituitary axis.[7] Moreover, adrenocorticotrophic hormone (ACTH), at physiological levels, injected into rats elevates plasma thymulin, whereas ACTH, administered *in vitro*, stimulates thymulin release from cultured thymic fragments.[8] Other hormones, including prolactin, growth, and thyroid hormones, also exert regulatory effects on thymulin release.[6] All these observations support the existence of a bidirectional communication between the immune and the nervous system, which is believed to represent an important homeostatic mechanism. Cytokines and other products of immunocompetent cells are known to play a crucial role as signaling molecules in these interactions.[9] During inflammation, interactions between the immune and the nervous system become more apparent. In this review, we provide further evidence that strongly supports the hypothesis that thymulin can interact with the nervous system either directly or indirectly, thus forming an afferent component in a neuroendocrine immune loop.

HYPERALGESIC ACTIONS OF THYMULIN

One of the first lines of evidence indicating that thymulin interacts with the nervous system was obtained in a series of experiments showing the hyperalgesic actions of this peptide. Various doses of thymulin were administered either via intraperitoneal (i.p.) or intraplantar (i.pl.) injections, and their effects

on pain-related behavior and cytokine levels were assessed. Intraperitoneal injection of low doses of thymulin resulted in both mechanical hyperalgesia, as assessed by the paw pressure (PP) test, and thermal hyperalgesia, as assessed by the hot plate (HP) and tail-flick (TF) tests. The effect was apparent at doses ranging between 20–150 ng.[10] Similar observations were obtained with i.pl. injections of thymulin (0.5 ng, 1 ng, 5 ng, and 10 ng), which resulted in a significant reduction in nociceptive thresholds, localized to the injected hind paw of the rats, as assessed by the different pain tests.[11]

Thymulin-induced hyperalgesia, of either somatic or visceral origin, was not accompanied by any signs of inflammation (redness, swelling, edema). It is interesting to note here that thymulin-induced hyperalgesia was significantly reduced by pretreatment with the tripeptide Lys-D-pro-val,[10] known to antagonize interleukin-1β (IL-1β) and prostaglandin-E2 (PGE2)–induced hyperalgesia.[12] On the other hand, lys-D-pro-thr, known to antagonize IL-1β-induced hyperalgesia,[13] was significantly less effective.[10]

Furthermore, thymulin-induced hyperalgesia was prevented by pretreatment with dexamethasone and indomethacin.[11] Subsequent evidence indicating an important role for PGE2 was obtained with experiments using the cyclooxygnase (COX) inhibitor meloxicam, with a preferential effect on COX-2.[14] Pretreatment with meloxicam produced a complete reversal of both mechanical and thermal hyperalgesia as assessed by the different pain tests. These results suggest that thymulin may be acting directly or indirectly on nerve terminals of the CNS. The results also indicate that PGE2 might be a key mediator in these actions.

Role of Proinflammatory Cytokines

We investigated the role of cytokines in thymulin-induced hyperalgesia. The effect of antitumor necrosis factor α (TNF-α), interleukin-1 receptor antagonist (IL-1ra), and antinerve growth factor (NGF) antiserum on thymulin-induced hyperalgesia was studied either with pretreatment with each of these reagents, 30 min before thymulin injection, or with a mixture of all three (the cocktail). Our results indicated that a mild reduction in thymulin-induced hyperalgesia was obtained by IL-ra, even when used at relatively high doses. On the other hand, anti-NGF antiserum, used at concentrations shown to be effective in reducing inflammatory hyperalgesia induced by complete Freund's adjuvant,[15] was only effective in reducing mechanical hyperalgesia as assessed by the PP test. A more effective reduction in thymulin-induced mechanical and thermal hyperalgesia was obtained by the anti-TNF-α antiserum, at doses used previously to neutralize endogenous TNF-α and resulting in the reduction of carrageenan-induced hyperalgesia.[16] These results demonstrate that each of these cytokines contribute, to some extent, to the thymulin-induced hyperalgesia. This assumption receives further support from the fact that pretreatment with a "cocktail" of the antisera and antagonists resulted in an almost complete

reversal of the thymulin-induced hyperalgesia. Further proof for the involvement of proinflammatory cytokines and other mediators was obtained with experiments in which thymulin injection both i.p. and i.pl. resulted in a significant elevation in the level of proinflammatory mediators NGF and PGE2 as measured by specific ELISA.[17] For the experiments in which thymulin was injected i.p. into a group of rats, the level of IL-1β, IL-6, TNF-α, NGF, and PGE2 was determined in the liver of these animals and was found to be significantly elevated as compared to saline-treated controls.[10] Similarly, in rats treated with thymulin (5 ng , i.pl.), there was a significant elevation in the level of IL-1β and NGF in the injected paw as compared to the noninjected paw.[11] When the effect of pretreatment with meloxicam was investigated, our results showed that the increased levels of IL-1β, TNF-α, and NGF, due to thymulin injection, recovered their control levels.

We can conclude, therefore, that the proinflammatory mediators play an important role in thymulin-induced hyperalgesia with PGE2 having a prominent role.

Potential Action of Thymulin on the Nervous System

Previous results provide evidence for a possible direct and dual action of thymulin on the peripheral (PNS) and central nervous systems (CNS).

On the one hand, it has been shown that the hyperalgesic effects of either i.p. or i.pl. injections of thymulin are almost abolished in rats neonatally treated with capsaicin.[18] Similar attenuation of thymulin-induced hyperalgesia (i.p.) was observed in rats subjected to either subdiaphragmatic vagotomy or to ablation of the vagal capsaicin-sensitive fibers (CSPA). The residual hyperalgesic effects of thymulin were attributed to either the mediation of thymulin effect by non-CSPA fibers or to a possible humoral action of thymulin on the CNS. On the other hand, more recently, the induction of fos-like immunoreactivity (FLI) in the dorsal horn of the lumbosacral spinal cord of rats subjected to i.pl. injections of thymulin has been reported.[19] Several lines of evidence are in favor of the hypothesis that the FLI induced by thymulin may involve other mechanisms than nociceptive processing[19]: (*a*) fos-labeled neurons showed a temporal and spatial distribution different from those observed following injection of endotoxin (ET) or other irritants; (*b*) morphine pretreatment reduced FLI induced by i.pl. injection of thymulin at a concentration of 4 mg/kg, twice the amount needed to abolish FLI and hyperalgesia induced by i.pl. ET injections[18,20]; (*c*) pretreatment with meloxicam, a cyclooxygenase inhibitor, reduced thymulin-induced FLI at concentration of 2 mg/kg, while a dose of 0.4 mg/kg was sufficient to produce a significant reduction of the hyperalgesia induced by similar injections of thymulin.[19]

All these data are in favor of the hypothesis of a possible action of thymulin on the PNS and the CNS, either directly or through PGE2-dependent mechanisms.

TABLE 1. Effect of PAT on cytokine upregulation by i.pl. injection of ET (1.25 μg)

Treatment	IL-1 (pg/hind paw)	IL-6 (pg/hind paw)	NGF (ng/hind paw)	TNFα (pg/hind paw)
ET (1.25 μg) i. pl.	2,850 ± 255	2,831 ± 285	23 ± 1.73	350 ± 50
PAT (25 μg) + ET (1.25 μg)	1,686 ± 266	1,158 ± 197	16.73 ± 2.70	104 ± 12
P-value	< 0.05	< 0.01	< 0.01	< 0.001

Levels of each cytokine and NGF in the skin tissues of the hind paws are shown of a separate group of five rats for the indicated treatment. The levels of mediators were measured at the time peak of the upregulation by ET, which corresponds to 1 h for TNF and 3 h for the other mediators.

ANALGESIC AND ANTI-INFLAMMATORY EFFECTS OF THYMULIN AND ITS ANALOGUE, PAT

Two models of ET-induced inflammatory hyperalgesia have been characterized during the last decade. The first was based on systemic inflammation induced by i.p. injection of high doses of ET,[21] and the observed hyperalgesia, fever, and illness were attributed to the upregulation of cytokine levels, especially IL-1β and PGE2.[22] The second described inflammatory hyperalgesia that was induced by local (i.pl.) injection of a small dose of ET in rats and mice.[23] Further investigations showed that this localized inflammatory hyperalgesia was preceded by significant upregulation of proinflammatory cytokines and NGF[24,25] and that pretreatment with either anti-inflammatorey drugs or anti-inflammatory cytokines (IL-10) prevented the ET-induced hyperalgesia.[24,25] The present study was based on the use of both models, and showed significant inhibitory effects exerted by thymulin and PAT on the ET-induced hyperalgesia and upregulation of cytokine levels (TABLE 1).

Effects of High Doses of Thymulin on Inflammatory Hyperalgesia

In a first series of experiments, the possible effects of thymulin on pain behavior in normal animals and in animals with localized inflammation were investigated. The classical tests for mechanical (PP test) and thermal (HP, and TF tests) hyperalgesia were used. Different groups of rats and mice were subjected to pain tests for three consecutive days before and 1 day after intraperitoneal (i.p.) thymulin injections (0.5, 1, and 2 μg in 100 μL saline). Baseline values of the various pain tests in thymulin-injected animals were comparable to those of saline-injected rats. In a second group of experiments, the animals also received i.p. injections of thymulin (0.5, 1, and 2 μg in 100 μL saline) prior to intraplantar (i.pl.) ET injections (1.25 μg). At this dose level, ET has been shown to induce local inflammation and hyperalgesia, which peaks at 9 h in rats and 24 h in mice and subsequently recovers by 24 and 48 h, respectively.[23]

Thymulin injections reduced, in a dose-dependent manner, the ET-induced mechanical and thermal hyperalgesia. It was thus concluded that thymulin in supraphysiological doses can reverse inflammatory hyperalgesia without altering pain thresholds in intact animals. Further experiments were carried out to investigate whether this analgesic effect of thymulin correlates with changes in the levels of the proinflammatory cytokines IL-1β and nerve growth factor (NGF), specially in view of the recent demonstrations of a major role for the neurotrophin NGF in mediating inflammatory hyperalgesia.[26,27] During ET-induced hyperalgesia there was a significant increase in the levels of IL-1β and NGF, and pretreatment with steroidal and nonsteroidal anti-inflammatory drugs reversed the ET-induced hyperalgesia and downregulated the levels of IL-1β and NGF.[25] Pretreatment of rats with thymulin (1 μg in 100 μg saline, i.p.) prior to ET injection, downregulated IL-1β and NGF levels in a manner comparable to that observed with the anti-inflammatory drugs.[28] These findings strongly suggest that the attenuation of hyperalgesia by high doses of thymulin involve the downregulation of the proinflammatory cytokines.

Effect of PAT on Inflammatory Hyperalgesia

In a further study, we analyzed the analgesic and anti-inflammatory actions of a synthetic peptide analogue of thymulin (PAT), which was initially synthesized with the potential for clinical applications as an immunomodulating agent.[29] To characterize the analgesic and anti-inflammatory actions of PAT, we used the two animal models of inflammation and hyperalgesia already described using i.pl. and i.p. ET injections, the aim of this study being to investigate whether PAT could affect the inflammatory hyperalgesia and to compare the efficacy of this molecule to that of steroids, NSAIDs, and peptides with known anti-inflammatory and antihyperalgesic actions.

PAT in a single dose, was effective in decreasing the level of several inflammatory mediators, which may prove to be an important effect not shown by other anti-inflammatory molecules. As an illustration, PAT exerted stronger inhibition of ET-induced hyperalgesia than K (D)PV or K (D)PT and at a dosage at least 10 times lower than that used for both peptides.

Another interesting aspect in the effects of PAT treatment was the amelioration of the symptoms of illness behavior induced by systemic injection of ET. This amelioration took the form of reversal of the hyperalgesia, improvement of the motor behavior, and prevention of febrile reactions to ET. Cumulative evidence has shown that ET does not cross the blood–brain barrier[30] and can induce the illness behavior and fever through PGE2-dependent mechanisms.[22] Therefore, the antifebrile effect of PAT can be attributed to the inhibition of COX-2 mechanisms leading to PGE2 formation. This assumption can be correlated with the observed downregulation of the levels of cytokine and PGE2, which were shown to be upregulated in the liver following systemic injection of ET. However, we cannot exclude possible action of PAT, like thymulin, on the

afferent nerve fibers involved in nociception and neuroimmune regulations.[18] Recent evidence demonstrates that PAT exerts strong inhibition of neuropathic manifestations, which are not necessarily the end product of inflammatory mechanisms.[31]

The observed antihyperalgesic action of PAT can be ascribed to its inhibitory effects on the inflammatory cascade through the downregulation of the levels of proinflammatory cytokines and NGF. This inhibition may be attributed, at least in part, to the inhibition of COX-2 mechanisms targeted by the NSAIDs. Thus, PAT may exert its antihyperalgesic effects, at least partially, as do NSAIDs, by reducing the inflammatory reactions.

ANTI-INFLAMMATORY EFFECTS OF THYMULIN-INJECTED INTRACEREBROVENTRICULARLY

Ample evidence implicates inflammatory processes in the development of a number of neurodegenerative diseases and demonstrates that neurons and microglia can serve as targets and/or sources for various cytokines, which are believed to be involved in the neuropathology.[32,33] An important aspect of brain injury is its association with increased production of cytokines.[34] Moreover, it is now established that many of these inflammatory molecules, commonly associated with the peripheral immune system, are also produced within the CNS.[35] The proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, have been shown to play important roles in the development of sickness behavior and to the ensuing hyperalgesia.[36]

Considerable research is currently directed at targeting proinflammatory mediators like cytokines and the transcription factors responsible for their expression as a possible novel therapeutic approach for inflammation-associated neurodegeneration.[38]

The following experiments were designed, first, to characterize the effect of i.c.v. injection of ET in awake rats by assessing its nocipeptide effect using the paw withdrawal (PW) and the HP tests and to determine the effects of pretreatment with thymulin on the observed hyperalgesia; second, to determine the changes in the concentrations of proinflammatory cytokines in different areas of the brain following i.c.v. injections of either thymulin alone, ET alone, or ET preceded by thymulin. Knowledge gained by this investigation could allow the use of the rat model in question to study neuroimmune interactions and to investigate the effect of substances that modulate factors contributing to neurodegeneration.

Injection of relatively small doses of ET (1 μg) was sufficient to produce early and significant reduction in the latencies of the nociceptive (PW and HP) tests, which recovered within 24 h. This effect was more pronounced on the HP than on the PW test, which suggests a centrally triggered hyperalgesia affecting more selectively the supraspinally coordinated behavior (i.e., HP test).

Furthermore, the observed hyperalgesia was not accompanied by other evident signs of sickness behavior including motor disturbances, fever, or diarrhea observed with the higher doses of ET or other proinflammatory agents injected either via the i.p. or i.c.v. routes.[35]

Significant increases in the concentrations of IL-1β, IL-6, and TNF-α in the brain were observed following i.c.v. injection of a relatively small dose of ET. Similar results have been reported following the injection of ET or other inflammatory agents either in the CNS or peripherally.[39,40] The presence of proinflammatory cytokines at increased concentrations has been considered as responsible for the observed illness-induced behavior in general[35,41] and for the increased nociceptive reactivity or hyperalgesia.[37]

Effect of Intracerebroventricular Injections of Thymulin on ET-Induced Hyperalgesia and Upregulation of Cytokine Levels

Intracerebroventricular administration of different doses of thymulin did not result in significant changes in pain-related behavior in rats or in the levels of IL-1β, IL-6, and TNF-α in different brain areas, with the exception of mild alterations of TNF-α level in the diencephalon.

However, pretreatment (i.c.v.) with different doses of thymulin (0.1, 0.5, and 1 μg) 20 min before the ET (1 μg) injection (i.c.v.) reduced, the ET- induced hyperalgesia in a dose-dependent manner. In addition, this pretreatment exerted differential effects on the upregulated levels of cytokines in the brain. Measured 3–4 h after ET injection, the levels of IL-1 were reduced in the cerebellum and hippocampus, but not in the diencephalon. IL-6 levels recovered their normal values in all the examined brain areas following thymulin pretreatment.

Thus, thymulin appears to play a protective role against inflammation in the CNS similar to the action previously described against the inflammatory effects of i.p. injection of ET.[42] This protective effect appears to be exerted directly at the level of the CNS since i.p. administration of equivalent or greater (1–5 μg) doses of thymulin failed to reverse the effects of i.c.v. injection of ET.

These results provide behavioral and immunochemical characterization of a rat model for intracerebral inflammation. Also, it demonstrates the neuroprotective role of thymulin in the CNS, which becomes evident during inflammatory states. Although the mode of action of this molecule in the brain is not totally elucidated, these results indicate a potential therapeutic use of thymulin as an analgesic and anti-inflammatory drug.

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