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The Anti-Inflammatory and Immunomodulatory Activity of Thymulin Peptide is NF-κB-Dependent and Involves the Downregulation of IκB-α

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Abstract The immunomodulatory activity of thymulin and related analogues *in vivo* is not well characterized in the CNS. We have previously provided evidence for an anti-inflammatory potential of thymulin in downregulating proinflammatory cytokines in a NF-κB-dependent mechanism *in vitro*. Furthermore, we have shown that intracerebroventricular (ICV) treatment with thymulin in the hippocampus (HC) reduced the nuclear localization and activation of NF-κB, an effect mediated by the IκB-α/*p*IκB- α pathway *in vivo*. ICV stereotaxic injection of endotoxin (ET) differentially upregulated the nuclear translocation /expression of NF-κB_1 (p50), NF-κB_2 (p52), RelA (p65), RelB (p68) and c-Rel (p75) in the HC. Pretreatment with thymulin followed by ET exposure reduced the nuclear translocation of NF-κB subunits. The anti-inflammatory effect of thymulin seems to be mediated via the IκB-α pathway since thymulin downregulated ET-induced phosphorylation of IκB-α. Stereotaxic pretreatment with synthetic peptide analogue of thymulin (PAT) reduced the nuclear translocation of NF-κB subunits, an effect mediated by downregulating the phosphorylation of IκB-α. EMSA revealed dose-dependent inhibition of NF-κB/DNA activation mediated by ICV ET. These results indicate that the anti-inflammatory effect of thymulin/PAT, mediated by IκB-α, is NF-κB-dependent and involves the downregulation of the nuclear translocation of various NF-κB subunits and their subcellular activation.

Keywords: *anti-inflammatory, intracerebroventricular, IκB, NF-κB, PAT, thymulin*

1. Introduction

NF-κB is one member of a ubiquitously expressed family of *Rel*-related transcription factors that serve as critical regulators of proinflammatory cytokines and immunomodulators [1]. Thymulin, a nonapeptide hormone secreted by the thymus essentially for regulating T lymphocyte differentiation and function, has major anti-inflammatory and immunomodulatory properties [2], thereby it provides an interface between neuroendocrine-immune communication systems [3,4].

Originally known as 'serum thymic factor' (Facteur Thymique Serique; FTS) [5], thymulin binds to a carrier protein and bioavailable cationic zinc (Zn^{2+}) to exert its modulatory actions [6]. The biological activity of thymulin is dependent on equimolar interaction with Zn^{2+}, whose bioavailability affects cellular immune functions under physiological and pathophysiological conditions [6,7]. Moreover, it has been shown that thymulin is capable of modulating proinflammatory cytokines *in vitro* and *in vivo* [8,9,10], providing an evidence for a novel anti-inflammatory propensity [11].

Two distinct epithelial populations in the thymus, first described by Bach in 1977, produce thymulin (also known as thymic factor [TF]) [2]. The hormone is believed to be involved in T-cell differentiation and enhancement of T and NK cell actions. Besides these rather paracrine or auto-organic effects on the thymus-dependent immune system, thymulin seems to have neuroendocrine effects as well. There exist bidirectional interactions between thymic epithelium and the hypothalamus-pituitary axis (HPA); for example, thymulin follows a circadian rhythm, and physiologically elevated adrenocorticotrophic hormone (ACTH) levels correlate positively with thymulin plasma levels and conversely [12,13].

Recently, a peptide analogue of thymulin has been synthesized, dubbed PAT, and it has been reported to have anti-inflammatory effects [14-16]. PAT was initially synthesized while recognizing the potential for clinical applications as an immunomodulating agent [17]. It has been reported that there is 'potent analgesic and anti-inflammatory actions' of PAT *in vivo* [16]. Furthermore, PAT may have intriguing anti-hyperalgesic effects as it was able to reduce pain in a model of neurogenic inflammation [15]. More importantly, preliminary results obtained in our laboratory indicate that the anti-inflammatory, immunomodulatory potential of thymulin/PAT may involve the NF-κB pathway [16].

Although not entirely elucidated, the underlying mechanisms of thymulin/PAT-mediated immunoregulation are not fully understood [18]. It has been reported that the effects of thymulin in downregulating an inflammatory

signal are mediated, at least in part, by modulating intracellular cyclic nucleotides [19,20,21]. In addition to the potent anti-inflammatory properties of thymulin, Zn^{2+} can synergistically downregulate a pro-inflammatory signal by reducing the release of inflammatory mediators and by acting as an antioxidant [22,23]. Of particular importance, Zn^{2+} is required in mediating antioxidant-dependent inhibition of the redox-sensitive NF-κB, a transcription factor essential to the expression of pro-inflammatory genes encoding cytokines and other inflammatory mediators [11,24-32]. In addition, a redox-responsive mechanism involving Zn^{2+} has been implicated in regulating the DNA-binding kinetics of NF-κB *in vitro* [33]. The aforementioned is corroborated by the observation that thymulin-mediated inhibition of ET-induced production of proinflammatory cytokines in the alveolar epithelium is mediated via the regulation of NF-κB. Furthermore, it is shown that ET-mediated regulation of NF-κB nuclear translocation/activation in the hippocampus (HC) is modulated by ICV treatment with thymulin, involving the IκB-α/*p*IκB-α pathway. The anti-inflammatory property assigned to PAT *in vivo* and its molecular ramifications, however, have yet to be unraveled and subsequently ascertained [1].

These observations, therefore, prompted the investigation whether PAT has an immunomodulatory potential in the hippocampus and, if any, what the relevant molecular pathways involved are. It is particularly shown that PAT differentially downregulates ET-induced ICV-mediated upregulation of the nuclear translocation of NF-κB subunits, an effect dependent, at least in part, on the canonical IκB-α/*p*IκB-α pathway. This inhibitory effect of PAT further extends to downregulating NF-κB activation in the hippocampus.

These results indicate that ICV injection of ET regulates the nuclear translocation and activation of NF-κB within specific compartments in the brain, an effect particularly localized to the hippocampus. PAT also attenuated the ET-induced response, with particular involvement of the transduction pathway implicating IκB-α, the major cytosolic inhibitor of NF-κB. It is concluded that peptide analogue of thymulin regulates an inflammatory signal within the hippocampus by acting as an immunomodulator in suppressing an inflammatory signal via a NF-κB-dependent mechanism, an effect mediated by regulating the IκB-α/*p*IκB-α pathway.

2. Materials and Methods

2.1. Chemicals and Reagents

Unless otherwise indicated, chemicals of the highest analytical grade were purchased from Sigma-Aldrich. Adult (200 – 250g) male *Sprague-Dawley* rats were used in this study. The animals were housed under optimum conditions of light and temperature with food and water *ad libitum* and kept, in groups of 4 – 5, during the period of the experiment in clear plastic cages with solid floors covered with 3 – 6cm of sawdust. All experimental procedures involving the use of live animals were reviewed and approved under the Animals (Scientific Procedures) Act, 1986 (UK).

At the level of thymulin analogue, PAT (Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asp) [15,16,17,18], which was custom synthesized by Quantum Biotechnologies Inc., Canada, was reconstituted in physiologic sterile saline and injected for treatments at the indicated concentrations. Different groups of rats received ET prepared from *Salmonella typhosa* (strain 0901) (Difco, Detroit, MC, USA) in sterile, physiological saline. For ICV injections, the regarded ET concentration was stereotaxically administered in volumes of 5 – 10µl.

2.2. Thymic Hormone Immunomodulatory Effect on ET-Induced NF-κB Expression – ICV Injections and Pretreatments

The carboxymethyl cellulose (1% CMC in 150mM NaCl) was used as a carrier in which thymic hormones and peptide analogues were administered [11]. Animals were stereotaxically monitored for accurate injections of ET intracerebroventricularly (ICV), with minimal stress under mild anesthesia. Animals were pretreated with thymulin (0.1 – 5µg; ICV) or peptide analogue of thymulin (PAT; 1 – 25µg; ICV) for 30 minutes prior to stimulation with ET (1µg) for 45 minutes, and simultaneously monitored before sacrificing and tissue extracting, essentially as reported elsewhere [10,34,35,36,37]. All pretreatments were carried out for brief periods to minimize neutralizing the effect of hormones following long exposure. This effect is also bolstered by using the CMC carrier. Although the experiments run for this study used CMC as a carrier, subsequent studies in our laboratory have shown that the modulatory effects of thymulin were not affected with or without CMC. Hence, subsequent experiments introduced thymulin without CMC. In the therein reported experiments, thymulin or PAT were not conjugated with Zn^{2+}, knowing that systemic circulating levels of Zn^{2+} may well contribute to the biological activity of thymulin [14,15,17]. However, *in vitro* experiments introduced thymulin and thymulin-Zn^{2+} conjugates where we have observed synergistic effect [1].

2.3. Cytosolic and Nuclear Protein Extraction for IκB and NF-κB Analysis Following Thymic Hormone Pretreatments

Cytosolic and nuclear extracts were prepared from tissues, essentially as reported elsewhere [26], with minor modifications. Samples were washed twice in 5ml ice-cold, O_2 pre-equilibrated phosphate buffered saline (PBS) and cells (≈ 1-2 x 10^7) were collected and centrifuged at 420*g* for 5 minutes at 4℃. Nuclei were released by re-suspending the pellet in 250 µl buffer A containing (in mM): 10 Tris-HCl (pH7.8), 10 KCl, 2.5 NaH_2PO_4, 1.5 $MgCl_2$, 1 Na_3VO_4, 0.5 dithiothreitol (DTT), 0.4 [4-(2-Aminoethyl)-benzene sulfonyl fluoride-HCl (AEBSF), and 2µg/ml each of leupeptin, pepstatin A and aprotinin. The suspension was left in ice for 10 minutes followed by a 45-second homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at 4500*g* for 5 minutes at 4℃ and re-suspending in 100µl buffer B (Buffer A adjusted to (in mM): 20 Tris-HCl (Ph 7.8), 420 KCl, 20% (v/v) Glycerol). The supernatant formed is

termed the cytosolic extract (used for IκB-α and *p*IκB-α analysis). The nuclei were then lysed at 4 ℃ for 30 minutes with gentle agitation, the debris cleared by centrifugation at 10000*g* for an additional 30 minutes at 4 ℃ and the supernatant frozen in liquid nitrogen and stored at −70 ℃ until used. In all cases, protein contents were determined by the Bradford method using BSA as a standard [24,26].

2.4. Western Analysis and Electrophoretic Mobility-Shift (EMSA) Assays

Cytosolic and nuclear proteins (20-25μg) were resolved by SDS-PAGE using a 7.5% separating phase at room temperature at 150V for 1h. After electrophoretic transfer onto nitrocellulose, each membrane was washed in Tris-buffered saline (TBS: 20mM Tris-HCl (pH7.6); 500mM NaCl) followed by blocking for 1h at room temperature in TBS plus 0.1% (v/v) Tween-20 (TBS-T) with gentle agitation. After three washes in TBS-T, membranes were incubated with mouse monoclonal IgG_1 anti-IκB-α (H-4), IgG_{2b} anti-*p*IκB-α (B-9), rabbit polyclonal IgG anti-p50 (NF-κB_1; NLS), anti-p52 (NF-κB_1; K-27), anti-p65 (RelA; A), anti-p68 (RelB; C-19), and anti-p75 (c-Rel; N) antibodies for primary detection (Santa Cruz Biotechnology, CA., USA; 1:500) in TBS-T overnight at 4 ℃. Primary conjugates were visualized on film using an anti-rabbit IgG-biotinylated antibody coupled with streptavidin-horseradish peroxidase enhanced chemiluminescence (ECL; Amersham Life Science). β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of β-actin was subsequently performed [24,26].

Nuclear extracts were analyzed for NF-κB DNA binding activity by electrophoretic mobility shift assay (EMSA) and supershift experiments with specific antibodies were performed as described previously (see below) [24,26]. Specific quantification of the corresponding DNA gel shift bands was performed with phosphorimaging.

EMSA was conducted using the following radiolabeled deoxy-oligonucleotide sequences purchased from Genosys: NF-κB (consensus sequence underlined) W-22: 5′-AGTTGAGGGGACTTTCCCAGGC-3′; (1bp missense control, M-22: 5′-AGTTGAGGCGACTTTCCCAGGC-3′). After end-labeling with polynucleotide kinase (Boehringer Mannheim), purifying and annealing probes, identical amounts of radioactivity (2 x 10^4 counts.min^{-1}) were added to binding reactions containing 1-5 μg nuclear extracts in a final volume of 40μl in DNA binding buffer (20mM HEPES (pH7.9); 1mM $MgCl_2$; 4% Ficoll) containing 0.15μg polydeoxyinosinic-deoxycytidylic acid [poly (dI-dC)] (Boehringer Mannheim) as a non-specific competitor. Mixtures were incubated for 30 minutes at 25 ℃ before separating on native non-denaturing 4% polyacrylamide gels at room temperature by electrophoresis in Tris-Borate-EDTA buffer. Where indicated, non-labeled oligonucleotide competitor was added in 100-fold molar excess immediately prior to addition of a radiolabeled probe of the same sequence.

2.5. Statistical Analysis and Data Presentation

Data are presented as means ± SEM of at least 3 independent experiments. Statistical evaluation of the difference in mean separation was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence level was considered at $P \leq 0.05$.

3. Results

3.1. Analysis of the Molecular Pathways Associated with the Effect of Thymulin on ET-Induced NF-κB Subunit Expression and Nuclear Translocation

In order to unravel the mechanism associated with the anti-inflammatory effect of thymulin in the CNS, varying concentrations of thymulin (0.1 – 5μg; ICV) were introduced stereotaxically 30 minutes before ICV injection of ET (1μg; 45 minutes). As shown in Figure 1, ET (Lane-3) upregulated the nuclear translocation of NF-κB_1 (p50), NF-κB_2 (p52), RelA (p65), the major transactivating member of the Rel family [25,27], RelB (p68) and c-Rel (p75) in the hippocampus (HC). The protein expression of NF-κB_1 (p50) and NF-κB_2 (p52) was mild as compared with other NF-κB subunits, with prominent expression of p65. Thymulin (5μg; ICV) alone (Lane-2) or control (Lane-1) experiments that received no injections showed faint constitutive or no expression, respectively, as compared with ET.

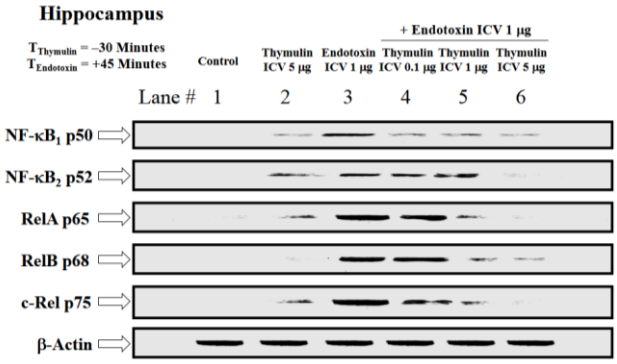

Figure 1. The expression of various NF-κB subunits in the hippocampus in response to ICV ET. Varying concentrations of thymulin (0.1 – 5μg; ICV) were introduced stereotypically 30 minutes before ICV injection of ET (1μg; 45 minutes). ET (Lane-3) upregulated the nuclear translocation of NF-κB_1 (p50), NF-κB_2 (p52), RelA (p65), RelB (p68) and c-Rel (p75) in the hippocampus (HC). The protein expression of NF-κB_1 (p50) and NF-κB_2 (p52) was mild as compared with other NF-κB subunits, with prominent expression of p65. Thymulin (5 μg; ICV) alone (Lane-2) or control (Lane-1) that received no injections showed faint constitutive or no expression, respectively, as compared with ET. The inductive effect of ET is ameliorated and attenuated with thymulin, particularly at concentrations ≥ 1 μg (Lanes-4, 5 and 6), with prominent effects on p52, p65 and p75 subunits. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. $n = 3 – 5$, which represents the number of independent experiments in separate animals

The inductive effect of ET is ameliorated and attenuated with thymulin, particularly at concentrations ≥ 1μg (Lanes-4, 5 and 6), with prominent effects on p52, p65 and p75 subunits. To ensure semi-quantitative loading per lane, the housekeeping protein β-actin was assayed by Western analysis relative to expression of various NF-κB subunits, as shown in Figure 1. Therefore, β-actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable.

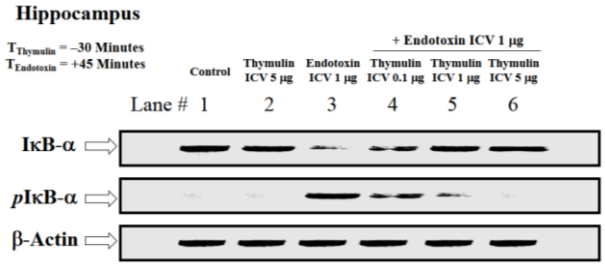

Figure 2. The effect of ET and thymulin pretreatment on IκB-α. Regulated by an upstream kinase (IKK), IκB-α is phosphorylated prior to the nuclear translocation of NF-κB. ET induced the phosphorylation of IκB-α, thereby tagging it for proteasome degradation, hence its cytosolic concentration was decreased (Lane-3). Thymulin, in a dose-dependent manner, reduced the phosphorylation of IκB-α, with maximal inhibitory effect at ICV concentration of 5μg (Lanes-4, 5 and 6). Thymulin restored IκB-α cytosolic accumulation, in a dose-dependent manner, consistent with the downregulation of its phosphorylation (Lanes-4, 5 and 6; Figure 2). Neither the control (Lane-1) nor thymulin alone (Lane-2) showed any effect on the phosphorylation of IκB-α, but there is constitutive expression as compared with ET alone. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. n = 3 – 5, which represents the number of independent experiments in separate animals

3.2. Analysis of the Effect of Thymulin on ET-Induced IκB-α Phosphorylation and Cytosolic Accumulation

Further assessment of the underlying mechanism pertaining to the inhibitory effect of thymulin on ET-induced nuclear localization of NF-κB subunits, the major cytosolic inhibitor of NF-κB was put into light. Regulated by an upstream kinase, dubbed IKK [12,36,37], IκB-α is phosphorylated prior to the nuclear translocation of NF-κB. ET induced the phosphorylation of IκB-α, thereby tagging it for proteasome degradation, hence its cytosolic concentration was decreased (Lane-3) (Figure 2). Thymulin, in a dose-dependent manner, reduced the phosphorylation of IκB-α, with maximal inhibitory effect at ICV concentration of 5 μg (Lanes-4, 5 and 6). Of note, thymulin restored IκB-α cytosolic accumulation, in a dose-dependent manner, consistent with the downregulation of its phosphorylation (Lanes-4, 5 and 6; Figure 2). Neither the control (Lane-1) nor thymulin alone (Lane-2) showed any effect on the phosphorylation of IκB-α, but there is constitutive expression as compared with ET alone. The β-actin distribution depicts semi-quantitative loading in parallel lanes.

3.3. Analysis of the Molecular Pathways Associated with the Effect of PAT on ET-Induced NF-κB Subunit Expression and Nuclear Translocation

As shown in Figures 3A and 3B, ICV ET (1μg) induced the expression of various NF-κB subunits (Lanes 1 and 2), albeit differentially (see Figure 1). No tangible effect on the nuclear expression of NF-κB subunits is shown with PAT alone (ICV; 1–25μg) (Lanes 3–5). PAT differentially reduced the nuclear localization of NF-$κB_1$ (p50), RelA (p65), RelB (p68) and c-Rel (p75), but not NF-$κB_2$ (p52). The most prominent effect of PAT is on p65 and p68 subunits (Figures 3A and 3B). The β-actin distribution depicts semi-quantitative loading in parallel lanes.

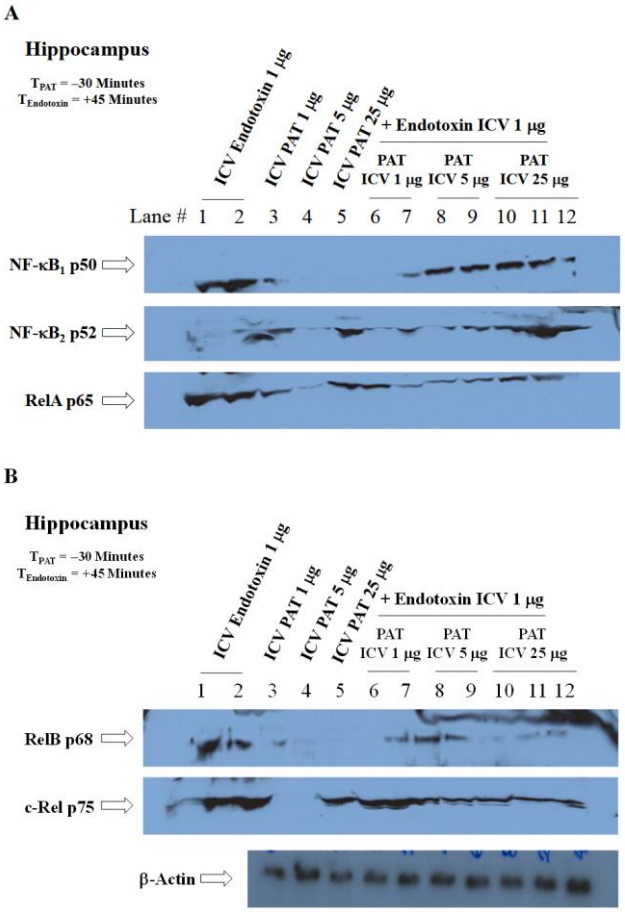

Figure 3. The modulation of the expression of various NF-κB subunits in the hippocampus in response to ICV ET/PAT. (***A***), (***B***) Varying concentrations of PAT (1 – 25μg; ICV) were introduced stereotaxically 30 minutes before ICV injection of ET (1μg; 45 minutes). ET (Lanes-1 and 2) upregulated the nuclear translocation of NF-$κB_1$ (p50), RelA (p65), RelB (p68), c-Rel (p75) and, to a lesser extent, NF-$κB_2$ (p52) in the hippocampus (HC). The protein expression of NF-$κB_2$ (p52) and RelB (p68) was mild as compared with other NF-κB subunits, with prominent expression of p65. PAT (1 – 25 μg; ICV) alone (Lanes-3-5) showed faint constitutive or no expression of NF-$κB_1$ (p50), NF-$κB_2$ (p52) and RelB (p68), as compared with ET. The inductive effect of ET is ameliorated and attenuated with PAT, particularly at concentrations ≥ 1μg, with prominent effects on p65 subunit. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. n = 3 – 5, which represents the number of independent experiments in separate animals

3.4. Analysis of the Effect of PAT on ET-Induced IκB-α Phosphorylation and Cytosolic Accumulation

The effect of PAT on the cytosolic accumulation of IκB-α is shown in Figure 4. PAT alone (1 and 25μg) exhibited cytosolic presence of IκB-α prior to ET or PAT

treatment, indicating constitutive expression. PAT pretreatment followed by ET upregulated the cytosolic accumulation of IκB-α (Figure 4). Whether this effect is mediated by the phosphorylation of IκB-α has been subsequently investigated. PAT alone (1 and 25μg) showed constitutive phosphorylation of IκB-α, an effect abrogated by PAT/ET, with prominent effect of PAT at 5μg (Figure 5). The β-actin distribution depicts semi-quantitative loading in parallel lanes (Data not shown).

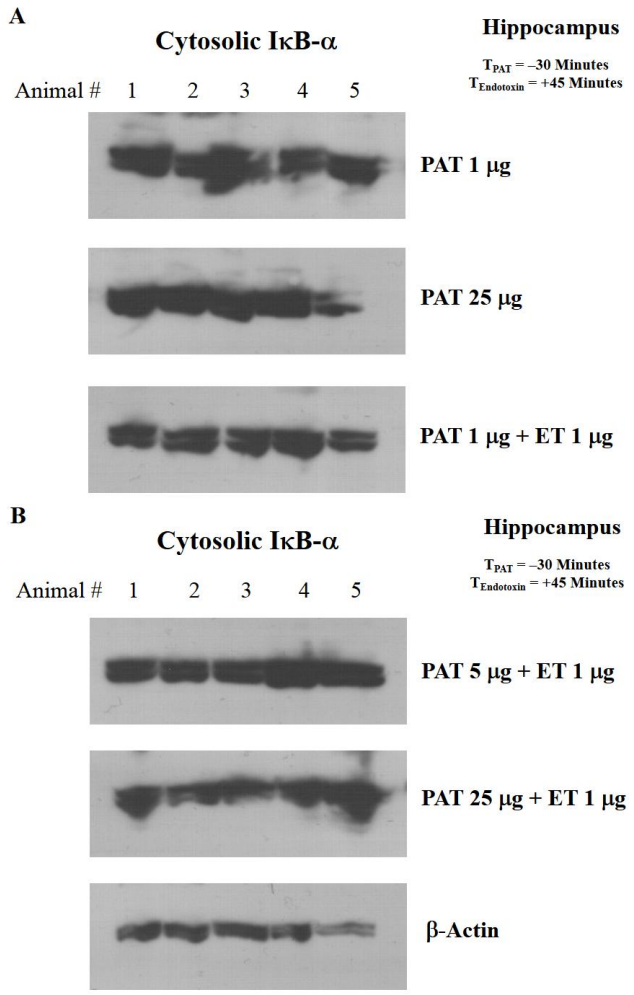

Figure 4. The effect of ET and PAT pretreatment on IκB-α. Regulated by an upstream kinase (IKK), IκB-α is phosphorylated prior to the nuclear translocation of NF-κB. ET induced the phosphorylation of IκB-α, thereby tagging it for proteasome degradation, hence its cytosolic concentration was decreased. (***A***), (***B***) PAT induced the cytosolic accumulation of IκB-α. PAT-mediated restoration of IκB-α cytosolic accumulation is, to an extent, dose-dependent. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. n = 3 – 5, which represents the number of independent experiments in separate animals

3.5. Analysis of the Effect of ICV ET and PAT Pretreatment on NF-κB DNA-Binding Activity – Dose Dependency

To ensure that the nuclear translocation of NF-κB is associated with DNA-binding activity, electrophoretic mobility shift assay was undertaken. As shown in Figure 6, PAT reduced, in a dose-dependent manner, NF-κB/DNA-binding activity, as compared with ET.

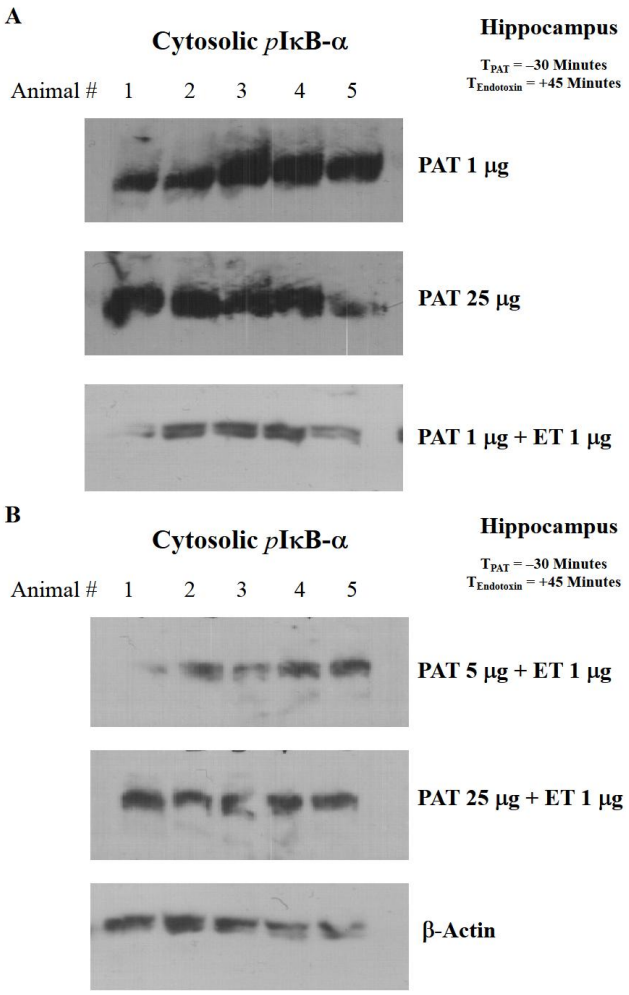

Figure 5. The effect of ET and PAT pretreatment on IκB-α phosphorylation. (***A***), (***B***) PAT reduced, in a dose-dependent manner, the phosphorylation of IκB-α, with maximal inhibitory effect at ICV concentration of 5 μg. This is consistent with restoration of IκB-α cytosolic accumulation (as shown in Figure 4). Neither PAT (1μg) nor PAT (25μg) alone showed any effect on the phosphorylation of IκB-α, but there is constitutive expression as compared with ET alone. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable (the β-actin for Lanes-1 and 2 [ICV ET] has not been reproduced in this experiment; however, it was shown in Figure 1 and Figure 2). n = 3 – 5, which represents the number of independent experiments in separate animals

4. Discussion

The present study shows that thymulin and its analogue exert a selective immunomodulatory influence on the *in vivo* regulation of ICV endotoxin-mediated expression of NF-κB subunits, nuclear translocation and DNA activation. Evidently, the anti-inflammatory effect of PAT is IκB-α-dependent, a pathway closely involved with the regulation of NF-κB [34-39].

We have previously shown that the immunomodulatory potential mediated by thymulin is exhibited by down-regulating an inflammatory signal through the differential reduction of the secretion of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α [8-11,16,34]. This selective inhibition is accompanied by upregulating an anti-inflammatory response mediated by IL-10 [11], consistent with a counter-inflammatory signaling loop amplified by thymulin [40]. This leads to suggest that

thymulin may exhibit an anti-inflammatory activity by acting as a novel dual regulator: It down-regulates a pro-inflammatory signal and, on the other hand, amplifies an anti-inflammatory response [1,2,11].

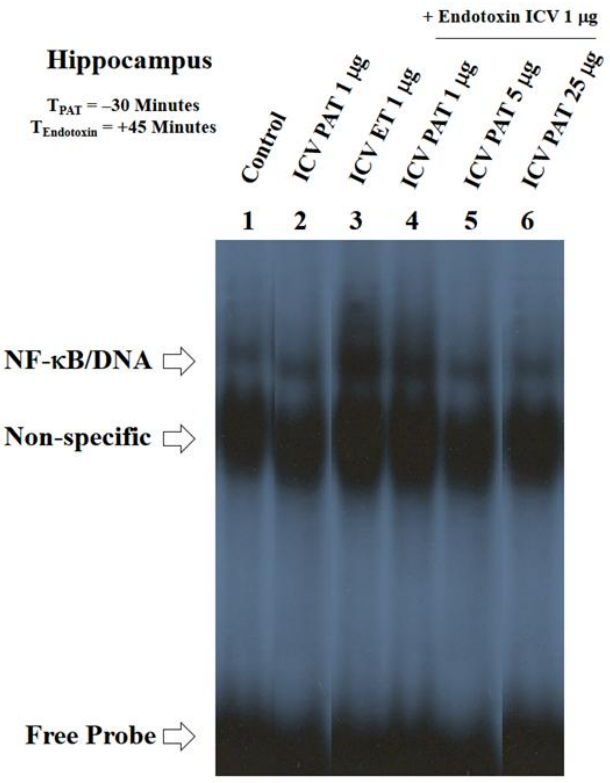

Figure 6. DNA-binding activity of NF-κB in response to ET/PAT. PAT reduced (Lanes-4-6), in a dose-dependent manner, ET-mediated NF-κB activation (Lane-3) in the hippocampus, as compared with saline (Control; Lane-1) or PAT alone (Lane-2), $n = 3 – 5$, which represents the number of independent experiments in separate animals

The immunomodulatory potential of thymulin/PAT has yet to be ascertained in terms of unraveling the cellular and molecular pathways associated with this effect in the CNS, and particularly in the hippocampus. Further and in sync with the aforementioned, thymulin and PAT may have the efficacy of down-regulating an inflammatory signal via the NF-κB pathway. Therein, it is reported that thymulin and PAT are capable of suppressing the nuclear translocation of various NF-κB subunits, notably RelA (p65), the major transactivating member of the Rel family [24,26,29]. Whether this inhibitory effect on the translocation of NF-κB is mediated via the nuclear localization sequence (NLS) has yet to be ascertained, but this jibes with the notion that the effect of thymulin on the NF-κB pathway is likely involving an upstream regulator, namely the IκB kinase (IKK). Preliminary results affirm this position in that thymulin can regulate the phosphorylation of IκB-α and IκB-β *in vitro* [1,9] and *in vivo* [1], an observation corroborated elsewhere [14,15,16]. Of particular significance is the observation that thymulin and PAT can modulate the NF-κB pathway via the down regulation of the nuclear translocation of various subunits, in addition to suppressing the DNA-binding activity.

The aforementioned results conspicuously affirm that thymulin can regulate the phosphorylation of IκB-α in the hippocampus, an observation corroborated elsewhere [4,9,11,37]. Of particular significance is the observation that thymulin can modulate the NF-κB pathway via the differential down regulation of the nuclear translocation of various subunits, in addition to downregulating their activation [1].

According to the best of my knowledge and after having carefully combed the MedLine literature for a potential involvement of the NF-κB in the regulatory effect of thymulin and PAT, I have observed only few references shedding albeit a faint light on the integral role of thymulin in regulating an anti-inflammatory response via the NF-κB pathway. For example, IKKβ$^{-/-}$ radiation chimeras exhibited elevated circulating TNF-α and IKKβ$^{-/-}$ thymocytes displayed increased TNF-α sensitivity, an early indicator for apoptosis [38]. This observation is reinforced with another report indicating a suppressor mechanism of thymulin on TNF-α-induced apoptosis in the mouse pancreatic β-cell line [41].

The compartmentalized immunomodulatory potential of PAT or thymulin in the CNS have yet to be ascertained in terms of unraveling the implicated cellular and molecular pathways [37,39]. Hence, deciphering the role of intracellular pathways may shed light on the mechanism of action of thymulin in the CNS. There is a wealth of data, however, that correlate the anti-inflammatory actions of thymulin with various mechanisms. For example, it was shown that the growth hormone-releasing activity of thymulin is receptor-mediated and involves calcium (Ca^{2+}), cAMP and inositol phosphates [21]. Furthermore, thymulin has been reported to modulate intracellular cAMP in pathophysiologic conditions [19].

Furthermore, and interestingly, that the observation that centralized actions of ET are counteracted by localized actions of thymulin (hippocampus) can suggest specificity in its actions, although we cannot rule out the influence of the animal model we're using in our laboratory for various behavioral studies [16]. However, what's evident is that ET induction of the NF-κB pathway is localized to the hippocampus and not any other area assessed in this study.

Retrospectively, the hippocampus is a major component of the brain where it belongs to the limbic system and hence plays important role in long-term memory and spatial navigation [37]. Pathophysiologically, for instance, in Alzheimer's disease the hippocampus is one of the first regions of the brain to suffer damage; memory problems and disorientation appear among the first symptoms. Damage to the hippocampus can also result from oxygen starvation (hypoxia), encephalitis, or medial temporal lobe epilepsy [37]. Whether the anti-inflammatory effect of thymulin/PAT can be neuroprotective beyond the anti-inflammatory conventional concept has yet to be ascertained, however [1].

Of note, ICV effect of ET and its reversal by thymulin/PAT is not only particularly localized to the hippocampus, but also not involving the diencephalon (DE) or substantia nigra (SN) [1]. The DE is known to be the part of the forebrain that contains such important structures as the thalamus, hypothalamus and the posterior portion of the pituitary gland [37]. The hypothalamus performs numerous vital functions, most of which relate directly or indirectly to the regulation of visceral activities by way of other brain regions and the autonomic nervous system [16,35]. However, this does not explain why systemic treatment with thymulin can reduce a central

inflammatory response localized to the CNS at specific compartments (hippocampus) [1,2,14,15,16]; whether that has major repercussions to the routes involved with the effect of thymulin is worth pursuing.

As that also applies to a non-responsiveness of the SN, I can theorize that given the fact that the brain is immunologically a privileged organ [37], the relative non-immune responsiveness of the brain has been attributed to a lack of lymphatic drainage, the presence of the blood-brain barrier (BBB), the lack of constitutive expression of the major histocompatibility complex (MHC) cluster and the presence of chemical mediators or cofactors purported as capable of inhibiting lymphocyte traffic during inflammation (neuronal cell death and inflammation) [1]. This evasion of systemic immunological recognition confers a privilege property that is so unique and, in many ways, plays a major role in shaping the grounds for modified neuroimmune interactions, and hence the observation of the rather limited role of an inflammatory signal or its counteraction by thymulin [37].

In corroboration with the above-mentioned, our group has previously shown that the sympathetic efferent fibers are technically involved with ET-induced localized inflammatory hyperalgesia and cytokine production in peripheral tissues, an effect abrogated by localized and systemic administration of thymulin [9,16,34,35]. It is likely that the bidirectional influence of neuroimmune interactions is a major factor in mediating the anti-inflammatory response of thymulin centrally and peripherally [37]. What is significant is the unequivocal involvement of the IκB/NF-κB pathway as an integral component of the immunomodulatory effectiveness of thymulin in regulating systemic and peripheral inflammation [37]. This is also reinforced with the observation that thymulin reverses inflammatory hyperalgesia and modulates the increased concentration of proinflammatory cytokines induced by ICV ET injection [10].

In coming back to PAT, I note that this peptide is synthetic. Combing the literature at MedLine revealed that the first time an analogue of thymulin was mentioned was in a report on graft rejection [42,43]. Interestingly, synthetic serum thymic factor (FTS) and several of its analogues have been examined for their immunoregulatory properties in a murine skin graft rejection model, including a syngeneic male-to-female system and an allogeneic system. More importantly, the analogue of thymulin accelerated or delayed rejection depending on dosage, indicating the multiplicity of action of thymic peptides on the various T cell subsets [42,43].

Following the aforementioned unconventional observation with the anti-inflammatory influence of thymulin, collaborative research at the American University of Beirut with CNRS in France led to the evolution of a peptide analogue of thymulin, dubbed PAT, which was later patented [14]. This reminds me to indicate that perhaps the efficacy of thymulin resides in a few key amino acids, but further site-directed mutagenesis is recommended to decipher the exact sequences of other analogues of thymulin that may exhibit similar, and potentially anti-inflammatory, effects. The anti-hyperalgesic and anti-inflammatory effects of PAT can be attributed, at least partially, to the downregulation of proinflammatory mediators [9,16]. This observation was taken another level with PAT to indicate that its anti-inflammatory influence may involve analgesic modulation of neurogenic pain [14,15]. Furthermore, the results therein reported have unequivocally shed light on the emphatic involvement of the NF-κB/IκB-α pathway, which in and of itself may constitute a foundation for further elaborating on the cellular and molecular pathways regulated by thymulin and its analogues.

5. Conclusions and Prospects

In summary, this report presents a novel immunomodulatory potential of thymulin and its analogue in the hippocampus. Furthermore, the selectivity of the immunomodulatory effect of thymulin/PAT accentuated its central mechanism via the NF-κB pathway. The results are highlighted as follows: *i*) thymulin exhibits a differential and selective, yet localized, inhibition of the NF-κB pathway; *ii*) stereotaxic localization led to specific intracerebroventricular injection of ET onto the CNS, with or without pretreatment with thymulin; *iii*) treatment with ET upregulated the expression and nuclear localization of NF-κB_1 (p50), NF-κB_2 (p52), RelA (p65), RelB (p68) and c-Rel (p75) in the hippocampus (HC), an effect abrogated, in a dose-dependent manner, by ICV pretreatment with thymulin; *iv*) thymulin modulated the phosphorylation of IκB-α in the HC by upregulating the cytosolic accumulation of IκB-α and downregulating *p*IκB-α; *v*) the DNA-binding activity of RelA (p65) was upregulated in the HC, an effect reduced by thymulin; *vi*) PAT reproduced the immunomodulatory inhibitory effects of thymulin by abrogating ET-induced NF-κB nuclear translocation and activation, a mechanism accentuated by regulating the phosphorylation and cytosolic accumulation of IκB-α.

This exhibition of an immunomodulatory potential of thymulin and its analogue bears clinical consequences for understanding the pathways associated with inflammatory signals. The molecular regulation of thymulin via the NF-κB pathway in the CNS is critical to understanding the alleviating anti-inflammatory role of this nonapeptide, a major player in neuroimmune communications [1,37].

Disclosure of Interest

The authors declare that they have no conflicts of interest concerning this article.

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