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Thymulin and zinc (Zn^{2+})-mediated inhibition of endotoxin-induced production of proinflammatory cytokines and NF-κB nuclear translocation and activation in the alveolar epithelium: Unraveling the molecular immunomodulatory, anti-inflammatory effect of thymulin/Zn^{2+} *in vitro*

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ABSTRACT

The immunomodulatory potential of thymulin and zinc (Zn^{2+}) in the perinatal alveolar epithelium is not well characterized. In an *in vitro* model of fetal alveolar type II epithelial cells (FATEII), we have investigated the exhibition of an anti-inflammatory activity of this peptide hormone. Thymulin selectively ameliorated, in a dose-dependent manner, the endotoxin (ET/LPS [lipopolysaccharide])-induced release of IL-1β, but not IL-6 or TNF-α. Furthermore, Zn^{2+}, an anti-inflammatory antioxidant, which is required for the biological activity of thymulin, independently reduced the secretion of IL-1β, TNF-α and, to a lesser extent, at a supraphysiologic dose (1 mM), IL-6. The underlying cellular and molecular pathways associated with the anti-inflammatory effect of thymulin and Zn^{2+} in the alveolar epithelium are not well established. Further in this study, the role of cyclic AMP (cAMP) in the anti-inflammatory effect of thymulin was investigated, in addition to unraveling the possible involvement of the NF-κB pathway. Interestingly, thymulin upregulated, in a dose- and time-dependent manner, the release of the nucleotide cAMP. To understand whether the inhibitory effect of thymulin on cytokine release is cAMP-dependent, Forskolin, a labdane diterpene known to elevate intracellular cAMP, was shown to reduce the secretion of IL-1β and TNF-α, but not IL-6, an effect mimicked by dibutyryl-cAMP (dbcAMP), an analog of cAMP. Alveolar epithelial cells treated with thymulin markedly showed a downregulation of the nuclear translocation of RelA (p65), the major transactivating member of the NF-κB family, in addition to NF-$κB_1$ (p50) and c-Rel (p75), an effect mildly substantiated with Zn^{2+}. Furthermore, thymulin/Zn^{2+} reduced, in a dose-dependent manner, the DNA-binding activity of NF-κB (RelA/p65). These results indicate that the anti-inflammatory effect of thymulin, which is mediated by cAMP, is NF-κB-dependent and involves the downregulation of the release of proinflammatory cytokines, particularly IL-1β, an effect synergistically amplified, at least in part, by Zn^{2+}. The molecular regulation of thymulin via a NF-κB-dependent pathway is critical to understanding the anti-inflammatory alleviating role of this nonapeptide in regulating proinflammatory signals.

1. Introduction

Thymulin, a nonapeptide hormone secreted by the thymus for regulating T lymphocyte differentiation and function, is reported to have major immunomodulatory actions (Bach et al., 1977), thereby providing an interface between neuroendocrine-immune communication systems (Dardenne, 1999; Millington and Buckingham, 1992). Originally known as 'serum thymic factor' (*Facteur Thymique*

Abbreviations: ACTH, Adrenocorticotrophic hormone; AEBSF, Aminoethyl benzene sulfonyl fluoride; BSA, Bovine serum albumin; CMC, Carboxymethyl cellulose; CNS, Central nervous system; dbcAMP, Dibutyryl-cAMP; MTT, Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTT, Dithiothreitol; DMEM, Dulbecco's modified eagle medium; EMSA, Electrophoretic mobility shift assay; ELISA, Enzyme-linked immunosorbent assay; EC, Energy charge; ECL, Enhanced chemiluminescence; FTS, Facteur thymique serique; FATEII, Fetal alveolar type II epithelial cells; FCS, Fetal calf serum; HBSS, Hank's balanced salt solution; HPA, Hypothalamus-pituitary axis; NF-κB, Nuclear factor-κB; NLS, Nuclear localization sequence; ANOVA, One-way analysis of variance; PAT, Peptide analog of thymulin; PBS, Phosphate buffered saline; PDI, Phosphodiesterase inhibitor; TF, Thymic factor; Zn^{2+}, Zinc.

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Serique; FTS) (Dardenne et al., 1980), thymulin binds to a carrier protein and cationic zinc (Zn^{2+}) to exert its modulatory properties (Dardenne et al., 1993). FTS is essentially a biologically inactive nonapeptide, whereas thymulin, a nonapeptide, consists of FTS coupled in an equimolecular ratio to Zn^{2+} (Bach et al., 1977), which confers biological activity to the molecule (Dardenne, 1999). Hence, the term 'thymulin' is integral for the FTS-Zn^{2+} metallopeptide.

Therefore, the biological activity of thymulin is dependent on equimolar interaction with Zn^{2+}, whose bioavailability affects cellular immune responses under physiological and pathophysiological conditions (Dardenne et al., 1993; Prasad, 1998). Moreover, it has been shown that thymulin is capable of modulating proinflammatory cytokines in disease *in vitro* and *in vivo* (Safieh-Garabedian et al., 1993, 1996, 2003), providing an evidence for a novel anti-inflammatory potential (Haddad et al., 2000a).

Thymulin, also known as *thymic factor* (TF), is produced by two distinct epithelial populations in the thymus, initially described by Bach et al. in 1977. 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 the thymic epithelium and the hypothalamus–pituitary axis (HPA); for instance, thymulin follows a specific circadian rhythm, and physiologically elevated adrenocorticotrophic hormone (ACTH) levels correlate positively with thymulin plasma levels and vice versa (Hadley et al., 1997; Haddad et al., 2002a).

Recently, a new role for thymulin as an effector on proinflammatory mediators/cytokines has emerged. For example, a peptide analog of thymulin (PAT) has been reported to have analgesic effects at supraphysiologic concentrations and particularly neuroprotective anti-inflammatory effects in the central nervous system (CNS) (Dardenne et al., 2006; Saadé et al., 2003; Safieh-Garabedian et al., 2002).

The underlying mechanisms of thymulin-mediated immunoregulation are not fully understood (Reggiani et al., 2009). 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 (Mutchnick et al., 1982; Rinaldi-Garaci et al., 1985; Brown et al., 2000). In addition to the potent anti-inflammatory properties of thymulin, Zn^{2+} can synergistically downregulate a proinflammatory signal by reducing the release of inflammatory mediators and by acting as an antioxidant (Prasad, 2008). Of particular importance, Zn^{2+} was shown to be required in mediating the antioxidant-dependent inhibition of the redox-sensitive nuclear factor-κB (NF-κB), a transcription factor essential to the expression of proinflammatory genes encoding cytokines and other inflammatory mediators (Haddad et al., 2000a,b, 2001, 2002b,c,d; Prasad, 2007, 2008; Haddad, 2002a,b; Haddad and Land, 2000, 2002). In addition, a redox-responsive mechanism involving Zn^{2+} has been implicated in regulating the DNA-binding kinetics of NF-κB *in vitro* (Yang et al., 1995). The anti-inflammatory property assigned to thymulin/Zn^{2+} in the perinatal lung, however, has yet to be ascertained.

These observations, therefore, prompted the investigation whether thymulin/Zn^{2+}, separately or in conjugation, may have an immunomodulatory potential in the alveolar epithelium and the relevant ostensibly implicated molecular pathways. It is particularly shown that thymulin differentially downregulates the endotoxin-induced secretion of proinflammatory cytokines (IL-1β, IL-6 and TNF-α), and that this effect is accompanied by enhancing an antagonistic, anti-inflammatory response through an IL-10 sensitive pathway (Haddad et al., 2000a). The immunomodulatory, anti-inflammatory property of thymulin is partially amplified in a synergistic manner by Zn^{2+} which, on its own, non-selectively suppressed the release of proinflammatory mediators.

In unraveling the cellular and molecular pathways associated with the immunomodulatory effect of thymulin, it is therein reported that thymulin modulates intracellular cAMP, an effect mimicked by Forskolin and dbcAMP, which are known cAMP analogues. Furthermore, thymulin and Zn^{2+} suppressed the nuclear localization of the transcription factor NF-κB (p65) and substantially downregulated its DNA-binding activity, an effect correlated with cAMP-dependent downregulation of the release of proinflammatory cytokines. It is emphatically concluded that thymulin regulates an inflammatory signal within the alveolar epithelium by acting as a dual immunoregulator in enhancing the accumulation of intracellular nucleotides and subsequently upregulating a counter-inflammatory pathway by reducing the release of proinflammatory cytokines in a NF-κB-dependent mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise indicated, chemicals of the highest analytical grade were purchased from Sigma–Aldrich. Adult (200–250 g) pregnant female *Sprague–Dawley* rats were used in this study for the extraction of fetal alveolar cells. 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 3–6 cm of sawdust. All experimental procedures involving the use of live animals were reviewed and approved under the Animals (Scientific Procedures) Act, 1986 (UK).

2.2. Primary cultures of alveolar epithelia

Fetal alveolar type II epithelial cells (FATEII) were isolated from lungs of fetuses at day 19 of gestation, essentially as reported elsewhere (Haddad and Land, 2000; Haddad et al., 2000b). Epithelia were harvested and grown (5×10^6) at 152 Torr (≈21% O_2) and 37 °C for 24 h in serum-free PC-1 media. It has been regarded that the adenylate energy charge, an index of cell viability and competence, remained ≥0.7, and transepithelial monolayer resistance was monitored constant at ≥250–300 Ω cm^2 (Haddad and Land, 2000; Haddad et al., 2000b; Baines et al., 2001).

In further experimental details, fetal rats were removed from pregnant *Sprague–Dawley* mothers by caesarean section at day 19 of gestation (term = 22 days), the lungs excised, teased free from heart and upper airway tissue, and were finely minced then washed free of erythrocytes using sterile, chilled Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution (HBSS) (0.5 ml/fetus). The cleaned lung tissue was re-suspended in 1 ml/fetus HBSS containing trypsin (0.1 mg/ml), collagenase (0.06 mg/ml) and DNase I (0.012%, w/v) and was agitated at 37 °C for 20 min. The solution was then centrifuged at $100 \times g$ for 2 min to remove undispersed tissue, the supernatant was saved to a fresh sterile tube and an equal volume of Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal calf serum (FCS) was added to the supernatant. After passing the supernatant through a 120 μm pore sterile mesh, the filtrate was centrifuged at $420 \times g$ for 5 min, the pellet re-suspended in 20 ml DMEM/FCS and the cells were placed into a T-150 culture flask for 1 h at 37 °C to enable fibroblasts and non-epithelial cells to adhere. Unattached cells were washed three times by centrifugation at $420 \times g$ for 5 min each and then seeded onto 24 mm diameter Transwell-clear permeable supports (Costar; 0.4 μm pore size) at a density of 5×10^6 cells per filter and were allowed to adhere overnight at fetal distal lung pO_2 (23 Torr ≈ 3% O_2/5% CO_2).