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VIP limits LPS-induced nitric oxide production through IL-10 in NOD mice macrophages

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Abstract

The spontaneous non obese diabetic (NOD) mouse model of Sjögren's syndrome provides a valuable tool to study the onset and progression of both autoimmune response and secretory dysfunction. Vasoactive intestinal peptide (VIP) is a neuro and immunopeptide with prosecretory effect in salivary glands and anti-inflammatory actions in various models of autoimmune disease. Our purpose was to analyze the response of peritoneal macrophages to an inflammatory stimulus during the decline of salivary secretion in NOD mice and the potential anti-inflammatory effect of VIP. We present evidence of an increased nitric oxide production by peritoneal macrophages of NOD mice in basal and lipopolysaccharide (LPS)+IFN-γ-stimulated conditions and a lower IL-10 response to LPS compared with normal BALB/c mice. VIP inhibited LPS-induced TNF-α, IL-12 and nitrites accumulation in NOD macrophages while it increased IL-10 production. VIP effect was prevented by an anti-IL-10 monoclonal antibody and it showed an additive effect on exogenously added IL-10 only in NOD mice. The inhibitory effect of VIP-induced IL-10 on nitrites was mediated by COX metabolites mostly in NOD cells as indomethacine inhibited both the increase in IL-10 and the reduction of nitrites exerted by VIP. We conclude that both PGE2 and VIP inhibit nitric oxide production and increase IL-10 induced by LPS in NOD macrophages and VIP effect is mediated through an increase of COX metabolites and IL-10.
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Keywords: NOD mice; Macrophages; VIP; Nitric oxide; IL-10; PGE2

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder of unknown aetiology characterized by a severe dryness of the mouth and the eyes [1–3]. An aberrant activation of glandular epithelial cells has been proposed in the induction and perpetuation of the inflammatory response [4]. The spontaneous non obese diabetic (NOD) mouse model of SS provides a valuable tool to study the onset and progression of both the autoimmune response and the secretory dysfunction which are almost impossible to monitor in humans. As reported in patients, there is a poor correlation between the moderate sialadenitis and the deep reduction in saliva secretion in NOD mice [5] suggesting that the initial trigger of autoimmune exocrinopathy of NOD mice may reside in a defect in salivary gland homeostasis [6,7]. In line with this, we described a loss of nitric oxide synthase (NOS) activity in salivary

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glands and a reduced salivary secretion upon vasoactive intestinal peptide (VIP) stimulation in NOD mice developing SS-like symptoms [8–11].

VIP is a neuro and immunopeptide that promotes secretion in glandular epithelium [12,13], contributes to vasodilation and promotes anti-inflammatory effects and Th2 cytokine production [14,15]. VIP inhibits NOS induction and IL-12 while stimulates IL-10 in normal mouse activated macrophages *in vitro* [16]. It also showed an efficient control of inflammation when given *in vivo* to models of rheumatoid arthritis, Crohn's disease and diabetes [17–20]. Our goal was to investigate the effect of VIP on lipopolysaccharide (LPS)-activated macrophages of prediabetic NOD mice. We present evidence to support that macrophages from prediabetic NOD mice with signs of salivary dysfunction produce higher nitrite and lower IL-10 levels upon an inflammatory stimulus. We also showed that both PGE2 and VIP inhibit nitric oxide production and increase IL-10 induced by LPS in NOD macrophages and VIP effect is mediated through an increase of COX metabolites and IL-10.

2. Materials and methods

2.1. Animals

NOD and BALB/c female mice were bred and maintained in the Central Animal Care facility at the School of Exact and Natural Sciences, University of Buenos Aires. 16-week-old mice were fasted overnight with water *ad libitum* before used. They were routinely tested for blood glucose levels (Wiener Lab., Rosario, Argentina) and considered pre-diabetic as their values of serum glucose on two occasions over a 24-h period did not significantly differ from those of control mice (1.0± 0.1 g/l, n=23). All studies were conducted according to standard protocols of the Animal Care and Use Committee of the School of Exact and Natural Sciences, University of Buenos Aires.

2.2. Macrophage isolation

NOD mice of 16 weeks of age presenting a lower (<40%) saliva flow as compared with BALB/c control mice [11] were used for macrophage isolation. Peritoneal exudate cells were obtained by peritoneal lavage with ice-cold RPMI 1640 medium. Cells containing lymphocytes and macrophages, were washed twice and resuspended in ice-cold RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Rockville, MD), and were seeded in flat-bottom 96-well microtiter plates (Corning Glass, Corning, NY) at 5×10^5 cells per well in a final volume of 200 µl. The cells were incubated at 37 °C for 2 h to adhere, and non-adherent cells were removed by repeated washing with RPMI 1640 medium.

Macrophage monolayers (>95% macrophages) were stimulated with 10 µg/ml LPS alone or together with 100 U/ml IFN-γ in the presence or absence of VIP (Neosystem, France), IL-10 (BD; San Diego, CA, USA) or PGE2 (Sigma; St Louis, MO, USA) were added from the beginning of the incubation time at

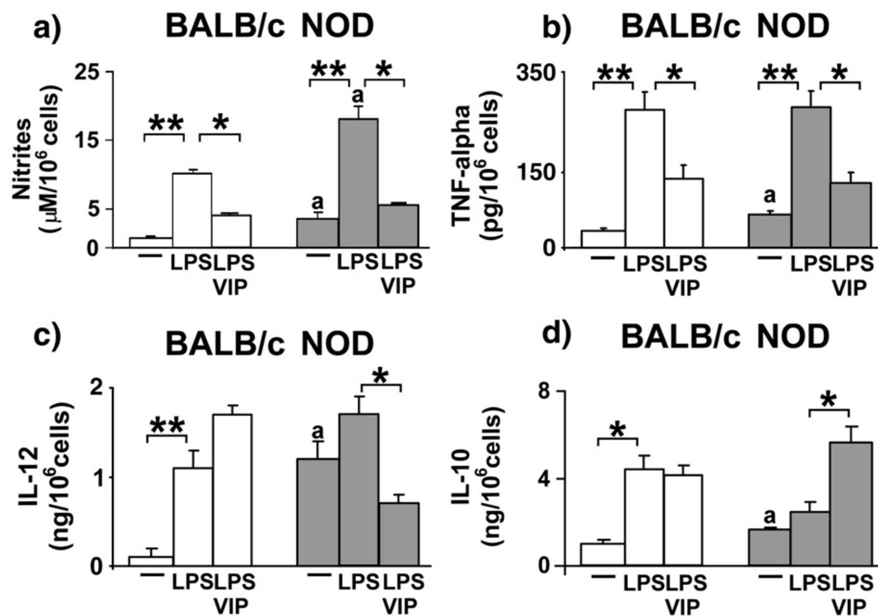

Fig. 1. *Higher inflammatory response of NOD mice macrophages:* Isolated macrophages from NOD (dashed bars) and BALB/c (empty bars) were treated with LPS+IFN-γ in the presence or absence of VIP (10^{-7} M) and supernatants were collected for cytokine and nitrite determination as described in Materials and methods. Results are mean±S.E. of at least 4 experiments. * $P<0.05$; ** $P<0.01$ vs. indicated bars (ANOVA). (a) means $P<0.05$ vs. corresponding condition in BALB/c (Student's t-test).

37 °C in a humidified incubator with 5% CO_2. When used, monoclonal antibodies (Pharmingen, San Diego, CA, USA) or indomethacine (Sigma, St Louis, MO, USA) were added 40 min before LPS. Cell-free supernatants were harvested at the designated time points and kept frozen until cytokine, nitrites or PGE2 determination.

2.3. Cytokine determination

Cytokines (IL-10 and IL-12) were determined in macrophage supernatants with a capture ELISA assay as previously described [11]. Briefly, microtitre plates (Corning Inc., New York, USA) were coated with a capture monoclonal anti-mouse IL-10 or IL-12 antibody (Pharmingen, San Diego, CA, USA) at 2 μg/ml at 4 °C. After washing and blocking with phosphate-buffered saline containing 3% bovine serum albumin, sera were added for 12 h. Unbound material was washed off and biotinylated monoclonal anti-IL-10, and anti IL-12 antibodies (Pharmingen) were added at 2 μg/ml for 45 min and revealed with avidin-peroxidase and ABTS substrate solution (Sigma, St Louis, MO, USA). The intra-assay and inter-assay variability for cytokine determination was <5%. The detection limits for serum samples were 15 pg/ml for IL-10 and 30 pg/ml for IL-12. TNF-α was determined by a bioassay on L929 cells treated with Actinomicin D [21]. After 20 h of incubation at 38.5 °C the cytolytic activity was determined by fixing and staining the cells and absorbance determined at 590 nm. The intra-assay and inter-assay variability for TNF-α determination was <15% and detection limit was 100 pg/ml.

2.4. Nitrites and PGE2 determination

Nitrite concentration was determined in macrophage supernatants obtained as described above for cytokine measurements using the Griess method with NEDA and sulfanilamide [22]. Results were expressed as μM of nitrites synthesized during 24 h/10^6 cells. For PGE2 determination, 100 μl of supernatants from 10^6 macrophages after 24 h incubation were frozen until RIA was carried out as previously reported [23].

2.5. Statistical analysis

Statistical significance of differences was determined by the two-tailed *t*-test for independent populations, especially when NOD vs. BALB/c values were compared. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. IC_{50} values were calculated with the LIGAND. Differences between groups were considered significant at $P<0.05$.

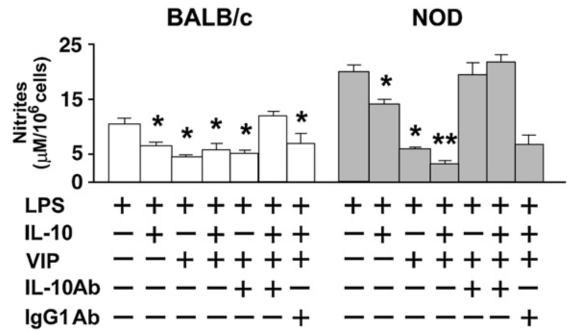

Fig. 2. *Effect of IL-10 and VIP on nitrite production:* Isolated macrophages from NOD (dashed bars) and BALB/c (empty bars) mice were treated for 24 h with LPS+IFN-γ in the presence or absence of VIP (10^{-7} M), IL-10 (75 U/ml), monoclonal Ab anti-IL-10 (IL-10Ab) (5 μg/ml) or a non-related IgG_1 antibody (IgG_1Ab) (5 μg/ml) and nitrites determined in the supernatants. Results are mean±S.E. of at least 4 experiments. * $P<0.05$; ** $P<0.01$ vs. LPS+IFN-γ within each mice group.

3. Results

3.1. Differential activation profile of NOD macrophages in response to LPS

NOD mice macrophages were stimulated with LPS alone or together with IFN-γ for assessment of nitrite and cytokine production. Fig. 1 shows that NOD macrophages produce higher nitrites, TNF-α, IL-12 and IL-10 concentrations than normal BALB/c cells in basal conditions. LPS+IFN-γ increased nitrites, TNF-α and IL-12 in NOD and BALB/c macrophages though with a higher effect on nitrites in NOD vs. BALB/c (Fig. 1a). A significant difference was observed in IL-10 production between NOD and control mice since LPS+IFN-γ was unable to increase this anti-inflammatory cytokine in the NOD (Fig. 1d). We asayed LPS alone (without IFN-γ) on nitrites to ascertain whether NOD peritoneal macrophages were already activated but there was no effect (data not shown).

Table 1
Effect of VIP on nitrites, IL-10 and PGE2 production

	Nitrites (μM/10^6 cells)		IL-10 (ng/10^6 cells)		PGE2 (pg/10^6 cells)	
	BALB/c	NOD	BALB/c	NOD	BALB/c	NOD
BASAL	1.2±0.1	4.3±0.3	1.0±0.1	1.6±0.2	372±31	122±13
LPS+IFNγ+VIP	1.1±0.1	3.8±0.2	1.6±0.7	5.9±0.3**	530±32*	320±28**

Isolated macrophages from NOD and BALB/c were treated with VIP (10^{-7} M) and supernatants were collected for nitrite, IL-10 or PGE2 determination as described in Materials and methods. Results are mean±S.E. of at least 4 experiments. * $P<0.05$; ** $P<0.01$ vs. basal in each mice group.

Table 2
Effect of PGE2 on LPS-activated cells

	Nitrites (μM/10^6 cells)		IL-10 (ng/10^6 cells)	
	BALB/c	NOD	BALB/c	NOD
LPS+IFN-γ	11.0±0.6	20.0±1.2	4.4±0.1	2.4±0.6
LPS+IFNγ+PGE2	7.7±0.8*	12.0±0.2**	4.5±0.2	8.1±0.3**

Isolated macrophages from NOD and BALB/c were treated for 24 h with LPS+IFN-γ in the presence or absence of PGE2 (5×10^{-8} M) and supernatants were collected for nitrites and IL-10 determination as described in Materials and methods. Results are mean±S.E. of at least 4 experiments. * $P<0.05$; ** $P<0.01$ vs. basal in each mice group.

3.2. Anti-inflammatory effect of VIP on NOD-stimulated macrophages

The effect of VIP was assayed on NOD mice macrophages stimulated with LPS+IFN-γ. VIP (10^{-7} M) reduced nitrite accumulation in both NOD and control cells although with a lower potency for NOD mice ($IC_{50}\times10^{-9}$ M BALB/c=1.3±0.1; NOD=5.8±0.1). TNF-α and IL-12 production was also reduced by VIP in NOD mice (Fig. 1b). As expected for an anti-inflammatory effect and reported for mouse normal macrophages [16], VIP increased IL-10 in NOD macrophages stimulated with LPS+IFN-γ to the same extent than in normal macrophages (Fig. 1d). Interestingly, VIP alone was enough stimulus to increase IL-10 only in NOD macrophages (Table 1). Also, VIP alone had no effect on nitrites but it did stimulate PGE2 synthesis, an autacoid associated with the autocrine regulation of inflammatory processes (Table 1).

3.3. Participation of IL-10 and PGE2 in the effect of VIP on NOD macrophages

The differential profile displayed by NOD cells (higher nitrites and failure to increase IL-10 levels in response to LPS+IFN-γ) together with the effect of VIP on IL-10 and PGE2 prompted us to investigate whether IL-10 and PGE2 had a role in the effect of VIP on nitrites. We first tested whether IL-10 added exogenously and/or VIP-induced IL-10 took part in the inhibitory effect of VIP on nitrites. We chose to test a concentration of IL-10 (75 U/ml) that was equally effective to reduce nitrites in both BALB/c and NOD cells, as derived from a concentration–response curve performed with that purpose (not shown). Fig. 2 shows that exogenous IL-10 inhibited LPS+IFN-γ-induced nitrite production in NOD macrophages to a similar extent than in control cells. An additive inhibitory effect of 10^{-7} M VIP and IL-10 on nitrites was observed only in NOD mice (Fig. 2). To assess whether VIP reduced nitrites through the production of IL-10 in NOD mice, we repeated the experiments in the presence of a monoclonal anti-IL-10 neutralizing antibody (IL-10Ab) and a non-related IgG_1 (IgG_1-Ab) antibody as a control. Fig. 2 shows that anti-IL-10 antibody prevented the inhibitory effect of IL-10 in both mice but it prevented the effect of VIP only in the NOD macrophages.

With the aim of investigating the role of cyclo-oxygenase (COX) metabolites in the inhibitory effect of VIP, we first determined the effect of PGE2 (5×10^{-8} M) on nitrites and IL-10 in NOD and control cells. Table 2 shows that the concentration of PGE2 that decreased nitrites in LPS+IFN-γ-treated NOD cells was also able to increase IL-10. However, this was not seen for BALB/c macrophages where PGE2 did not change IL-10 levels. To further explore the involvement of COX metabolites in the effect of VIP in NOD macrophages, we tested the cyclo-oxygenase inhibitor indomethacine on IL-10, PGE2 and nitrites production. Fig. 3b shows that indomethacine prevented the effect of VIP on IL-10 in NOD cells and reversed the inhibitory effect of VIP on nitrites (Fig. 3a). The effect of the simultaneous

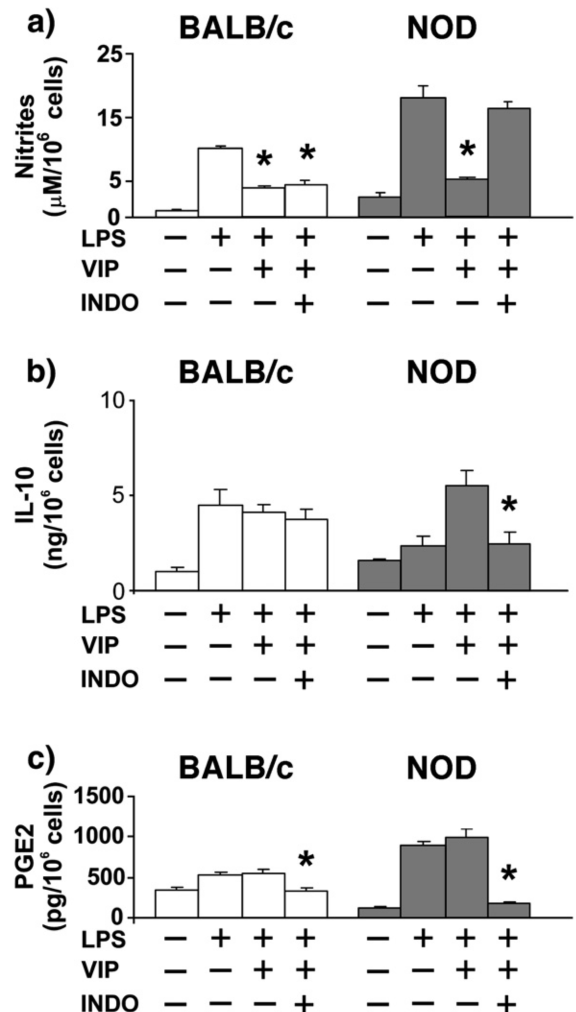

Fig. 3. *Involvement of cyclo-oxygenase products in the effect of VIP:* Isolated macrophages from NOD (dashed bars) and BALB/c (empty bars) mice were treated for 24 h with LPS+IFN-γ in the presence or absence of VIP (10^{-7} M) and indomethacine (10^{-6} M) (INDO) and nitrites, IL-10 and PGE2 determined in the supernatants as described in Materials and methods. Results are mean±S.E. of at least 4 experiments. In (a): * $P<0.05$ vs. LPS+IFN-γ within each mice group. In (b, c): * $P<0.05$ vs. LPS+IFN-γ+VIP.

addition of anti-IL-10 antibody and indomethacine on nitrites was similar to that of each inhibitor alone (data not shown).

4. Discussion

An altered cross-talk between the immune and neuroendocrine systems might have a role in the loss of homeostatic control that precedes autoimmune diseases like Sjögren's syndrome. However, attempts to disclose such early events and their mechanisms in chronic autoimmune disorders are limited mostly to experimental models such as the NOD mouse model where early biochemical alterations in target tissues and overlapping activation of committed immune cells can be followed up. Similar to SS patients, it has been suggested that the initial trigger of autoimmune exocrinopathy in NOD mice may reside in a defect of salivary gland homeostasis or signalling [6,7,10,11].

Results presented here indicate that macrophages from prediabetic NOD mice undergoing salivary dysfunction present some differences in their profile of activation as they are less effective than normal mice cells to regulate nitrites production when faced to an inflammatory stimulus, and that VIP acts through IL-10 and COX metabolites to limit nitrite production in NOD macrophages. Our conclusions are based on three main observations: First, NOD macrophages produce higher levels of pro-inflammatory IL-12, TNF-α and nitrites in basal conditions. Accordingly, NOD macrophages produced more nitrites than control cells when primed with LPS+IFN-γ and did not respond with an increase in the anti-inflammatory cytokine IL-10. Second, VIP inhibited LPS+IFN-γ-stimulated nitrites, IL-12 and TNF-α production and increased IL-10 in NOD mice cells. VIP alone increased IL-10 and PGE2 in both mice. Third, the effect of VIP to inhibit nitrite production was prevented by an anti-IL-10 antibody and it was additive to exogenously added IL-10 only in NOD mice. PGE2 increased IL-10 levels and decreased nitrites similarly to VIP and indomethacine inhibited the effect of VIP on both IL-10 and nitrites.

The mechanism that might underlie the loss of homeostatic control in NOD mice exocrinopathy is currently unknown and we have focused on the role of an early defect in nitric oxide synthase and VIP signalling in salivary glands of NOD mice developing SS-like symptoms [8–10]. This defect precedes the onset of the autoimmune reaction [11]. These observations collectively support the hypothesis of a pre-existing functional defect in the target organ that contributes to confer susceptibility to develop an autoimmune process. In this context, a defect in macrophage multiple regulatory pathways might be central to the pathogenesis of salivary dysfunction and recent reports on the diabetic stage of NOD mice show an impaired clearance of apoptotic cells and increased expression of cytokines by macrophages [24,25].

VIP appears as an interesting neuroimmune endogenous mediator in NOD exocrinopathy due to its pro-secretory action in salivary glands on one side [12,13], and as a modulator of the immune response with a selective anti-inflammatory action, on the other [14,15]. Certainly, VIP has been shown as a potent anti-inflammatory peptide in animal models of autoimmune diseases as well as in *in vitro* experiments with patient cells [17–20,26]. Its ability to promote IL-10 synthesis and to reduce iNOS induction and Th1 cytokine production promoted by LPS accounts for its anti-inflammatory effect *in vitro* and *in vivo* [14–16]. According to the present results, we hypothesized that the regulation of IL-10 on nitrites might give us some clues to the differential activation profile displayed by these cells. We also hypothesized that this might influence the mechanism of action of VIP in NOD macrophages and that – besides the already known pathways – there might be other mediators common to inflammatory responses and activatable by VIP such as COX products participating in the IL-10-iNOS interaction. Our results propose that a product of COX induced by VIP contributes to the effect of VIP to increase IL-10 and to decrease nitrites in NOD cells. The mutual regulation between the products of iNOS and COX-2 has been proposed in models of inflammation involving the action of IL-1β and IL-10 [27,28]. This might have a particular importance in NOD mice where a chronic inflammatory process affecting macrophages might condition both the activation profile and the regulation of their response. It is tempting to speculate that VIP-induced COX metabolites act in NOD cells in a complementary pathway by extending the effect of VIP-induced IL-10, for instance, by providing the cells with lipid metabolites more effective at the autocrine regulatory loops than IL-10. Evidence presented here on PGE2 effects and the ability of VIP to reduce nitrites through COX metabolites reflect subtle differences in the activation profile of NOD cells. This observation together with the need of including IFN-γ with LPS to stimulate macrophages suggest that changes in NOD macrophage profile do not appear as a drastic "all or none" situation but rather there are subtle differences that need to be approached mechanistically. This is probably the most common situation in the course of chronic diseases, where the significance and redundancy of the compensatory mechanisms is complex and variable. Certainly, the relative importance of the pathway presented here needs to

be further assessed in other models of inflammatory diseases that have been already used to study VIP effects. Finally, the results shown indicate that VIP alone was enough stimulus to increase IL-10 and PGE2 production in NOD macrophages. This is another specific difference between NOD and normal macrophages since it has been reported that VIP does not stimulate IL-10 production in macrophages unless LPS is present [16]. Also, VIP and IL-10 effects were additive only in NOD macrophages and anti-IL-10 neutralizing antibody prevented VIP effect only in NOD cells suggesting that this pathway to reduce nitrites is specially involved in NOD mice. Noteworthy, the concentration of IL-10 measured in the supernatants of LPS-activated NOD macrophages and VIP-treated NOD macrophages (~10 pg/μl) was in the same range of the effective concentration of exogenous IL-10 to reduce nitrites (75 U/ml, specific activity of this lot: $0.5–1.1 \times 10^7$ U/mg). IL-10 was shown to suppress NO and TNF-α produced by activated macrophages [29] and treatment with IL-10 reduced the severity of insulitis, prevented cellular infiltration of islet cells and normalized insulin production in NOD mice [30]. In addition, a high endogenous concentration of this cytokine is considered a positive prognostic marker in human autoimmune diseases [31]. Whether VIP has a role as an anti-inflammatory mediator *in vivo* for NOD exocrinopathy has to be proved yet, although it was reported as effective to reverse salivary dysfunction when given by means of a gene-transfer system [32]. Moreover, we have recently shown that pharmacological doses of VIP had an anti-inflammatory effect *in vivo* in diabetic NOD mice with an increase of seric IL-10 [19]. Disclosure of the mechanisms of VIP action including the role of IL-10 and COX metabolites in acute and chronic inflammation might offer potential strategies for combined therapeutic approaches.

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