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Activation of $VPAC_1$ receptors aggravates early atherosclerosis in hypercholesterolemic apolipoprotein E-deficient mice

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ABSTRACT

Objective: Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide widely expressed in the body and binding three types of receptors: $VPAC_1$-R, $VPAC_2$-R and PAC_1-R. Based on beneficial effects of VIP and $VPAC_1$-R agonists in mouse models of several chronic inflammatory disorders, we hypothesized that activation of VIP receptors would prevent atherosclerosis development in apolipoprotein E-deficient mice.

Methods and results: Contrary to our hypothesis, administration of a $VPAC_1$-R agonist, $(Ala^{11,22,28})$-VIP aggravated atherosclerotic lesion development in the aortic root of these mice compared to control mice. This was accompanied by a significant increase in the expression of MHC class II protein $I\text{-}A^b$, and suggests enhanced inflammatory activity in the vessel wall. The amount of macrophage-specific CD68 staining as well as serum cholesterol and triglyceride levels did not change as a result of the $(Ala^{11,22,28})$-VIP treatment, i.e. the treatment resulted in significant changes in lipid accumulation in the lesions without changing the number of macrophages or systemic lipid levels. Interestingly, administration of VIP did not alter the course of the disease.

Conclusion: Despite beneficial effects in murine models of several inflammatory disorders, $VPAC_1$-R activation aggravates atherosclerotic lesion formation in apolipoprotein E-deficient mice through enhanced inflammatory activity in the vessel wall.

1. Introduction and aims

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide, which is widely expressed in the nervous system [1] as well as by endocrine organs [2] and immune cells [3]. At physiologic concentrations VIP exerts most of its effects by binding two types of receptors: VIP/pituitary adenylate cyclase-activating peptide receptor type 1 and 2 ($VPAC_1$-R and $VPAC_2$-R, respectively) [4]. $VPAC_1$-R is constitutively expressed on macrophages and lymphocytes [5]. $VPAC_2$-R, on the other hand, is largely an inducible receptor appearing on macrophages and T-cells after stimulation [5]. VIP administration causes modulation of T-helper balance by suppressing Th1 immune responses. It inhibits leukocyte activity and migration, decreases nuclear factor-kappa B (NF-κB) activation and expression of pro-inflammatory cytokines, chemokines, as well as adhesion and co-stimulatory molecules, and decreases expression of coagulation factors and acute phase proteins (summarized in [3] and [6]). Many of these effects are mediated via $VPAC_1$-R. In light of these findings, it was proposed that VIP and other VPAC-R agonists may be beneficial in inflammatory disorders characterized by macrophage activation and misbalanced Th1/Th2 response. In fact, $VPAC_1$-R-mediated effects of VIP are protective in mouse models of other chronic inflammatory disorders such as rheumatoid arthritis [7], Crohn's disease [8], multiple sclerosis [9,10], and pancreatitis [11].

Atherosclerosis is a chronic disease characterized by a smoldering immune activation in focal areas of the vessel wall as well as local and systemic inflammatory changes [12]. Enhanced expression of adhesion molecules in the vessel wall, infiltration of immune cells (predominantly macrophages and T-cells), release of pro-inflammatory mediators (cytokines, chemokines, and leukotrienes) are all prominent features of this disease [12]. Consequently, therapeutic modulation of the immune response has been proposed to prevent development of new lesions and/or alleviate the

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burden of existing disease [13]. Since $VPAC_1$-R agonists are beneficial in the disorders with similar pathogenesis and inhibit the key pathological processes involved in atherogenesis, we hypothesized that exogenously administered VIP and a specific $VPAC_1$-R agonist would afford protection from atherosclerosis.

2. Materials and Methods

2.1. VIP and $VPAC_1$-R agonist and their delivery

VIP and the specific $VPAC_1$-R agonist ($Ala^{11,22,28}$)-VIP were purchased from Bachem AG (Bubendorf, Switzerland). Both agents were dissolved in sterile phosphate-buffered saline (PBS), which was also used as a vehicle for the control group of animals. Drugs were delivered with Alzet® osmotic minipumps (DURECT corporation, Cupertino, CA, USA) which continuously infused the substances at a rate of 5 nmol per day (equivalent to 16.6 μg/day for VIP and 15.8 μg/day for ($Ala^{11,22,28}$)-VIP) over a period of 4 weeks. Selection of the administered dose was based on previous studies examining the effects of VIP and specific $VPAC_1$-R agonists in murine models of rheumatoid arthritis [7], Crohn's disease [8], and multiple sclerosis [9,10].

Serum concentrations of VIP at the end of the experiment were analyzed using radioimmunoassay with VIP2 antiserum raised against conjugated natural porcine VIP. The detection limit of the assay was 3 pmol/L. The antiserum does not cross-react with gastrin, pancreatic polypeptide, glucagon, NPY or NT. Intra- and inter-assay coefficients of variation were 9% and 13%, respectively.

2.2. Experimental animals

Animal studies were approved by the regional ethical committee on animal research. Female apolipoprotein E-deficient ($ApoE^{-/-}$) mice backcrossed eleven times to C57BL/6 were purchased from Taconic M&B (Bomholtvej, Denmark) and fed regular chow and water *ad libitum*. At the age of 12 weeks, mice were implanted with Alzet® osmotic minipumps containing PBS, VIP or ($Ala^{11,22,28}$)-VIP (10 animals in each group). The pumps were implanted subcutaneously in the interscapular region and the skin wound was closed with metal clips. At the age of 16 weeks, the animals were sacrificed with carbon dioxide. Post-mortem, blood was collected immediately via cardiac puncture, and the animal was perfused with PBS, after which the heart and aorta (from ascending to iliac bifurcation) were microdissected *in situ*. The heart was immediately frozen in OCT compound (Tissue Teck, Sakura, Netherlands) for subsequent cryosectioning, while the aorta was preserved in 4% buffered formaldehyde solution for future *en face* lipid staining. The spleen was extracted for subsequent leukocyte isolation.

$ApoE^{-/-}$ mice fed regular chow and water *ad libitum* were used for RNA extraction of aortic tissue were. They were sacrificed at the age of 18 weeks and perfused with PBS after which aortas were microdissected *in situ* and immediately fresh-frozen at −80 °C.

2.3. RNA extraction and real-time PCR

RNA from mouse aortas was extracted with an RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA concentration and purity were assessed with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (0.5 μg) was reverse transcribed into complimentary DNA (cDNA) using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexanucleotide primers (Invitrogen). Quantitative RT-PCR was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) for mouse $VPAC_1$-R (catalogue number Mm00449214_m1), $VPAC_2$-R (Mm00437316_m1), and hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) (Mm00446968_m1). Results are shown in arbitrary units of expression relative to the standard curve obtained from 10-fold dilutions of mouse reference RNA (Stratagene, La Jolla, CA, USA) and normalized to the expression of HPRT.

2.4. Morphometric and immunohistochemical analysis of the lesions

To perform morphometric analysis of aortic root lesions the aortic root was cryosectioned over 800 μm above the level of the aortic valve cusps. Eight 10 μm sections per aortic root (one section at every 100 μm) were collected and fixed in 4% formaldehyde. Sections were stained with Oil-red O to aid microscopic detection of lesions. Images were captured to a computer with a Leica DC300 digital camera mounted on a Leica DMLB2 microscope and analyzed with the Leica QWin software (all from Leica Microsystems GmbH, Wetzlar, Germany). The atherosclerotic lesion area on each of the eight sections/aortic root was defined as the total lipid-stained area (μm^2) inside the internal elastic lamina of the vessel.

Formaldehyde-fixed aortas were cut open longitudinally, pinned out on wax pads and stained for lipids with Sudan IV (Merck AG, Darmstadt, Germany) [14]. Images were captured as described above. Ten aortas were evaluated in both the control and ($Ala^{11,22,28}$)-VIP-treated groups and nine were analyzed in the VIP-treated group.

To assess the composition of the lesions, acetone-fixed 10-μm-thick cryosections of the aortic root in the proximity of the largest lesion (measured as described above) were stained for structural, cellular and inflammatory markers. Staining for macrophages, T-cells and VCAM-1 was performed using rat-anti-mouse CD68 (Serotec Ltd., Oxford, UK), rat-anti-mouse CD3 (Southern Biotechnology, Birmingham, AL, USA) and rat-anti-mouse VCAM-1 (BD Pharmingen, San Diego, CA, USA) antibodies, respectively. For CD3 staining, 0.1% saponin in PBS was used to unmask epitopes. Stainings were developed with biotinylated rabbit-anti-rat IgG, biotin-avidin-peroxidase complexes (Vecstatin ABC kit, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB) (Vector Laboratories). $I\text{-}A^b$ expression was detected with biotinylated mouse-anti-mouse $I\text{-}A^b$ (BD Pharmingen), Vecstatin ABC kit and DAB. Expression of α-actin was visualized with alkaline-phosphatase-conjugated mouse-anti-mouse α-actin antibody (Sigma–Aldrich, St. Louis, MO) and Vector red development kit (Vector Laboratories). Slides were counterstained with hematoxylin to aid cell identification. Staining for collagen was performed with Sirius Red (Sigma–Aldrich). For CD68, α-actin, VCAM-1 and collagen, the total staining area was quantified at 100× magnification and expressed as % of total lesion area, while for CD3 and $I\text{-}A^b$, the number of positive cells in the section was counted at 400× magnification. All histological analyses were carried out in a blinded fashion by two independent observers.

2.5. Analysis of spleen lymphocyte subpopulations

Spleen mononuclear cells were isolated as previously described [15] and detected by flow cytometry using the following monoclonal antibodies (all from BD Pharmingen): FITC-hamster-anti-mouse TCRβ for total T-cells, FITC-hamster-anti-mouse TCRβ and PE-hamster anti-mouse CD69 for activated T-cells, FITC-rat-anti-mouse CD4 for $CD4^+$ cells, PE-rat-anti-mouse CD8a for $CD8^+$ cell and PE-rat-anti-mouse CD19 for total B-cells. Appropriate isotype control antibodies were used and apoptotic cells were excluded from the analysis by 7-AAD staining. Flow cytometry analysis was performed on a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.6. Analysis of serum lipoprotein fractions and cytokine levels

Serum was separated by centrifugation of clotted blood and stored at −80 °C until use. Total cholesterol and triglycerides were measured by standard enzymatic methods, while serum lipoprotein profiles were determined by fast protein liquid chromatography (FPLC) as previously described [16]. Serum levels of IL-10, MCP-1, IFN-γ, TNF-α, IL-12 and IL-6 were determined using the Cytokine Bead Array (CBA) Mouse Inflammation Kit (BD Pharmingen) on a FACSCalibur® flow cytometer.

2.7. Statistical analysis

Statistical analysis was performed with the StatView 5.0.1 (SAS institute Inc., Cary, NC, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) software packages. Comparison of aortic root lesion area was performed with ANOVA followed by Fisher's Least Significant Difference Test. All other comparisons were pre-specified comparisons of each treatment group with control group. For those, the two-tailed Mann–Whitney *U* test was used and *P*-values $\leqslant 0.05$ were considered statistically significant.

3. Results

3.1. A $VPAC_1$-R agonist increased atherosclerotic lesion size without affecting systemic lipoprotein fractions

Morphometric analysis of the aortic root atherosclerotic lesions from $ApoE^{-/-}$ mice performed at different levels (100–800 μm) above the aortic valve cusps revealed significantly larger lesions in mice treated with $(Ala^{11,22,28})$-VIP ($p < 0.05$), but not in those treated with VIP (all in comparison to control animals) (Fig 1A). Representative sections of aortic root stained with Oil-Red O are shown on Fig 1B–D. Analysis of the lipid content throughout the aortas stained *en face* with Sudan IV did not reveal significant differences among the three groups of mice, but showed a trend for more prominent lipid deposition in the aortas of $(Ala^{11,22,28})$-VIP-treated mice compared to control mice (Fig 1E) ($p = 0.08$).

Neither serum cholesterol nor triglyceride concentrations differed among the three groups of mice (Table 1) and cholesterol lipoprotein profiles were the same in all three groups (data not shown).

3.2. A $VPAC_1$-R agonist increased I-A^b expression

We assessed aortic root atherosclerotic lesions for several cellular, structural and immune activation markers using immunohistochemistry (Fig 2). In comparison to control mice, the number of I-A^b positive cells was significantly higher in $(Ala^{11,22,28})$-VIP-treated ($p = 0.04$), but not in VIP-treated mice (Fig 2A). There was no difference in the CD68- and α-actin-stained areas in $(Ala^{11,22,28})$-VIP-treated or VIP-treated animals compared to control mice (Fig. 2B and C). The number of CD3-positive cells and staining for VCAM-1 and collagen were not significantly different in either of the treatment groups compared with control mice (data not shown).

3.3. Expression of $VPAC_1$-R and $VPAC_2$-R in atherosclerotic lesions and serum VIP concentrations

To determine whether VPAC receptors are expressed in atherosclerotic lesions, we performed real-time quantitative PCR on RNA extracted from atherosclerotic aortas from $ApoE^{-/-}$ mice. Both $VPAC_1$-R and $VPAC_2$-R were present in the lesions and expression of $VPAC_2$-R was relatively higher than that of $VPAC_1$-R (Fig 3).

At the end of the 1 month-long infusion, VIP-treated animals showed a tendency for higher serum VIP levels compared to control animals (mean ± SEM: 9.9 ± 1.4 pmol/L in control animals and 13.0 ± 1.8 pmol/L in VIP-treated animals, p = NS). In each group, VIP levels were detectable only in 8 animals, while they were below detection limit of the assay in 2 animals. Circulating $(Ala^{11,22,28})$-VIP concentrations could not be measured since the anti-VIP antibodies used in the radioimmunoassay did not cross-react with $(Ala^{11,22,28})$-VIP.

3.4. VIP and $VPAC_1$-R agonist infusion did not affect splenic content of T- and B-cells

To determine whether administration of VIP or $(Ala^{11,22,28})$-VIP had a systemic effects on lymphocytes, flow cytometry analysis was performed on the homogenates of spleen tissues. The results revealed no differences in the total number of T-cells (TCR-β^+), activated T-cells (TCR-β^+CD69$^+$), CD4$^+$, CD8$^+$ cells, or B-cells (CD19$^+$).

3.5. VIP and $VPAC_1$-R agonist infusion did not affect systemic cytokine concentrations

Cytokine Bead Array (CBA assay) performed on serum samples revealed no differences in serum concentrations of IL-10, MCP-1, IFN-γ, TNF-α, IL-12 and IL-6.

4. Discussion

This study is the first to directly investigate effects of activation of VIP receptors on the development of early atherosclerotic lesions. Our study shows that administration of the specific $VPAC_1$-R agonist, $(Ala^{11,22,28})$-VIP [17], to $ApoE^{-/-}$ mice aggravates lesion development in the aortic root, but not in the abdominal aorta. At the same time, VIP infusion does not affect developing atherosclerosis at either site. These findings are unexpected as VIP and $VPAC_1$-R agonists have previously been shown to protect against a number of inflammatory disorders [7–11]. VIP has also been ascribed beneficial effects in other forms of cardiovascular pathology, including myocardial fibrosis [18,19] and cardiomyopathy [20], while some studies found a direct association between low VIP plasma levels and adverse prognosis in acute myocardial infarction [21] and chronic heart failure [22]. Such beneficial effects, however, were not observed in our mouse model of atherosclerosis.

The effects of $VPAC_1$-R activation on the immune system are not fully understood. Several studies show that activation of $VPAC_1$-R by VIP and $(Ala^{11,22,28})$-VIP suppresses a number of potentially pro-atherogenic events such as leukocyte activation and migration, NF-κB activation, expression of pro-inflammatory cytokines, chemokines, and adhesion molecules [3,6]. At the same time, activation of $VPAC_1$-R may elicit the opposite effects, including activation of monocytes and polymorphonuclear cells [23–25] and increase in production of IFN-γ by antigen-activated T-cells [26]. Our results in atherosclerotic mice are consistent with the latter effects since lesions from $(Ala^{11,22,28})$-VIP-treated mice were significantly larger and expressed more MHC class II protein I-A^b compared with control animals. I-A^b is normally not present on most vascular cells, but it is known to be induced by IFN-γ, which is produced locally as a consequence of immune activation in the lesion [27], and suggests enhanced cellular activation in the vessel wall. As $VPAC_1$-R activation has been reported to increase IFN-γ production by T-cells [26], this may be the mechanism responsible for the increased expression of I-A^b in the lesions of our mouse model.

Importantly, serum cholesterol and triglyceride levels remained similar between the treatment groups, which precludes influence

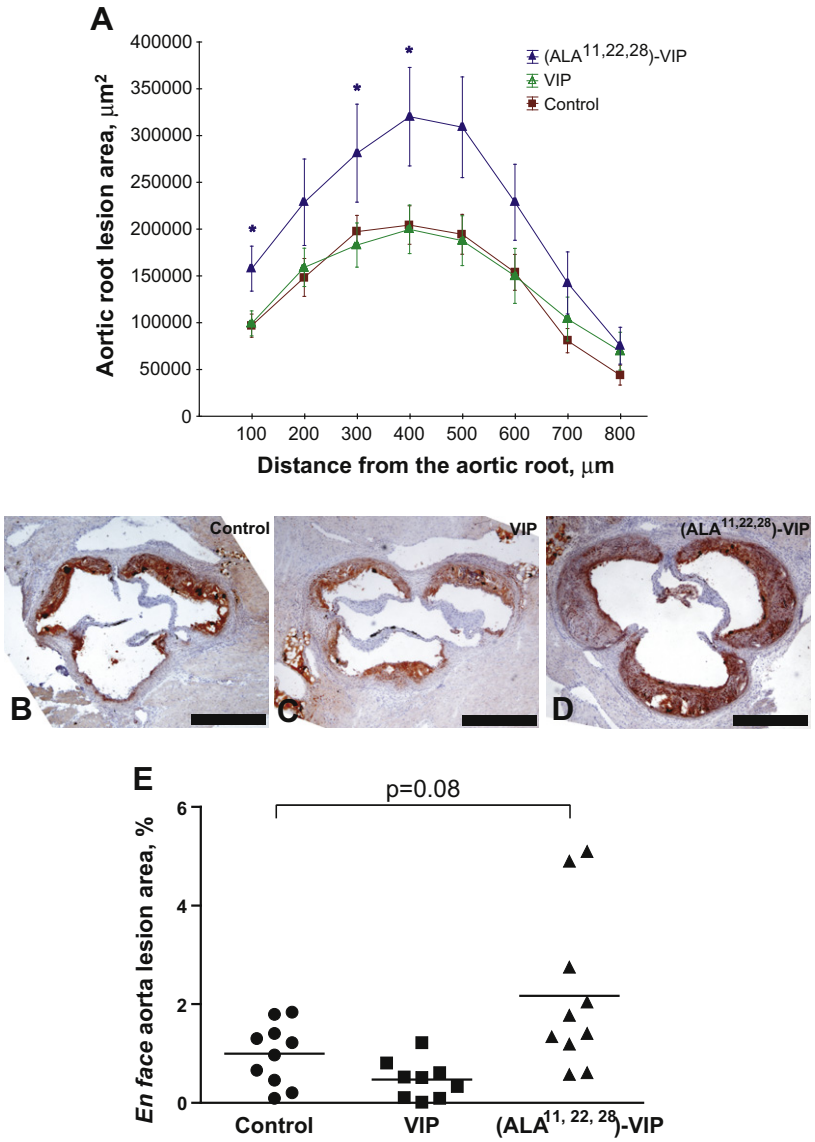

Fig. 1. Effects of VIP and (Ala^{11,22,28})-VIP on the formation of atherosclerotic lesions. (A) Aortic root lesion area (μm^2) in vehicle-treated (n = 10), VIP-treated (n = 9), and (Ala^{11,22,28})-VIP-treated (n = 9) ApoE$^{-/-}$ mice. Data on the graph are presented as mean ± SEM. Asterisks denote $p < 0.05$; (B–D) Oil-Red O stainings of representative aortic root sections from each group. Micron bars correspond to a distance of 300 μm; (E) Fraction (%) of the lipid deposition in the aortas determined by *en face* staining with Sudan IV in vehicle-treated (n = 10), VIP-treated (n = 9), and (Ala^{11,22,28})-VIP-treated (n = 10) ApoE$^{-/-}$ mice. Horizontal lines represent mean values.

Table 1
Serum cholesterol and triglycerides concentrations in vehicle-treated (n = 10), VIP-treated (n = 10), and (Ala^{11,22,28})-VIP-treated (n = 9) ApoE$^{-/-}$ mice. Data presented as mean ± SEM.

	Vehicle (n = 10)	VIP (n = 10)	(Ala^{11,22,28})-VIP (n = 9)	p value
Cholesterol, mM	13.8 ± 0.3	13.4 ± 0.4	14.0 ± 0.3	NS
Triglycerides, mM	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	NS

of different systemic lipid levels on atherogenesis in our study. This effect is noteworthy, since lipoproteins are one of the major pro-atherosclerotic agents and promote lesion expansion also in this mouse model [28]. The amount of macrophages detected by CD68 staining in the lesions from (Ala^{11,22,28})-VIP-treated mice was also unchanged compared to control mice. This suggests that (Ala^{11,22,28})-VIP treatment affects lipid accumulation in the lesions independent of significant changes in the number of macrophages – cells which are known to accumulate the majority of lipids in the plaques. Similarly, (Ala^{11,22,28})-VIP treatment did not affect the number of CD3$^+$ cells in the lesions or lesion stability estimated by the amount of smooth muscle α-actin staining.

While the specific VPAC$_1$-R agonist (Ala^{11,22,28})-VIP had significant effects on the size and immunological status of the developing atherosclerotic lesion, VIP administration did not affect atherosclerosis. In contrast to (Ala^{11,22,28})-VIP, full length VIP binds VPAC$_2$-R in addition to VPAC$_1$-R [29]. Our data show that atherosclerotic lesions express both types of receptors. Thus, lesions can bind both VIP and (Ala^{11,22,28})-VIP. However, differences in cell distribution, regulation, and functions of VPAC$_1$- and VPAC$_2$-receptors [29,30] may contribute to differential effects of these molecules. In particular, VPAC$_1$-R stimulation by a specific agonist has been shown to result in a 20-fold increase in INF-γ secretion from activated mouse T-cells [26]. This important finding strongly supports our study, as we observed significant increase in lesional expression of I-A^b known to be induced by IFN-γ. Therefore, we conjecture that VPAC$_1$-R activation, in this case (Ala^{11,22,28})-VIP treatment, can promote IFN-γ production, while the net result of activation of all VIP-

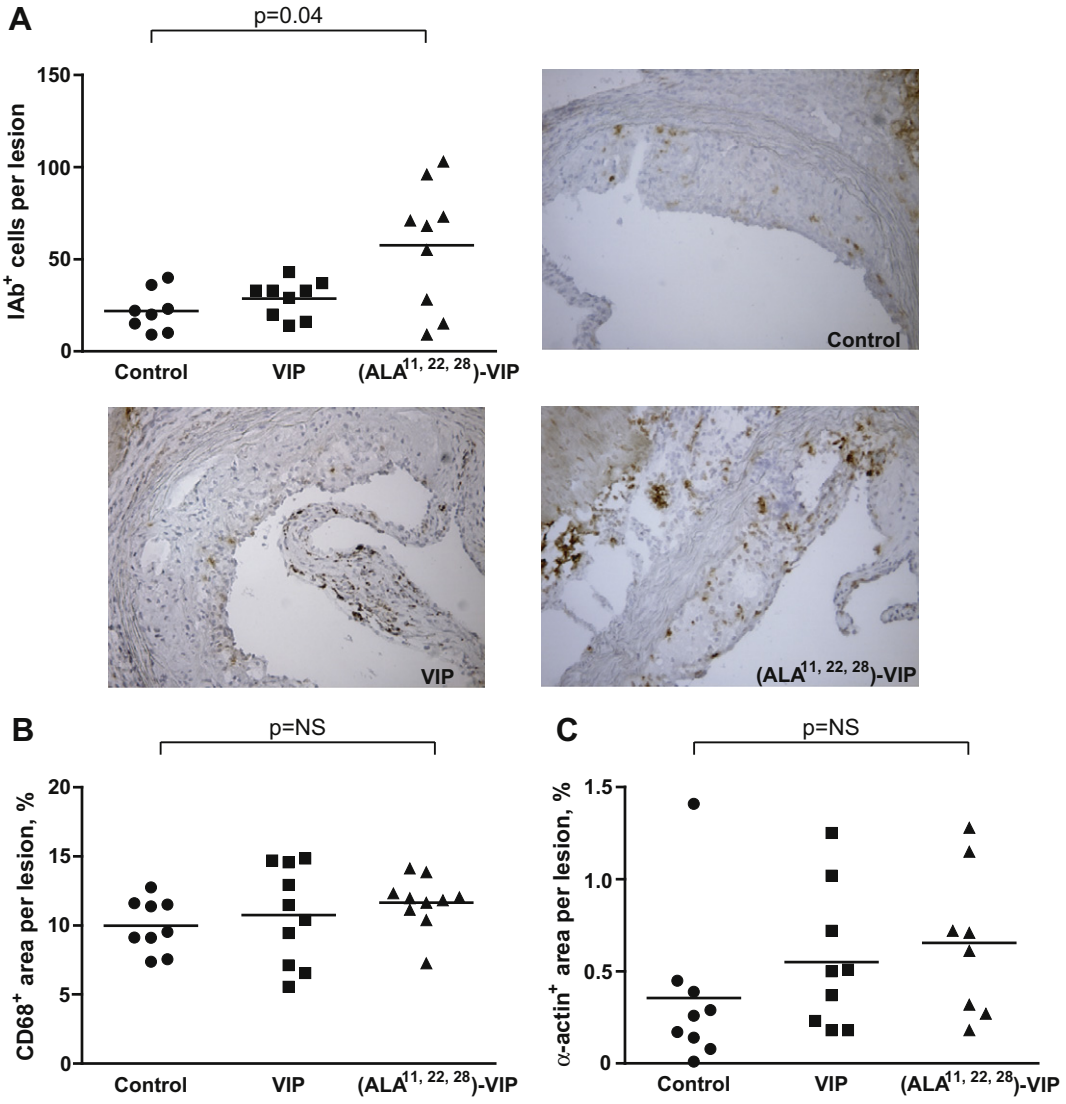

Fig. 2. Effects of VIP and ($Ala^{11,22,28}$)-VIP on the composition of atherosclerotic lesions. (A–C) Cellular and activation markers in aortic root lesions of vehicle-treated ($n = 9$), VIP-treated ($n = 10$), and ($Ala^{11,22,28}$)-VIP-treated ($n = 10$) $ApoE^{-/-}$ mice assessed by quantitative immunohistochemistry. Horizontal lines represent mean values for the number of I-A^b-positive cells in the lesions (A) or the fraction of the lesion area stained for α-actin and CD68 (B and C). Micrographs (A) show representative images (magnification × 200) of immunohistochemical stainings for I-A^b in each of the treatment groups. NS = not significant.

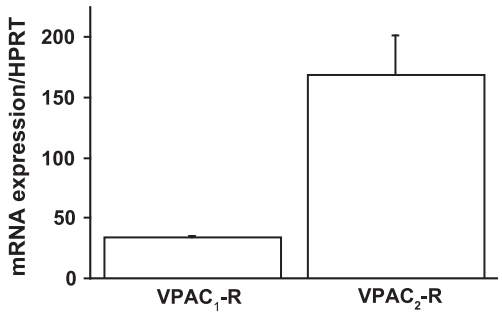

Fig. 3. Expression of $VPAC_1$-R and $VPAC_2$-R in atherosclerotic mouse aortas. Relative expression of $VPAC_1$-R and $VPAC_2$-R mRNA in mouse atherosclerotic aortas ($n = 12$) determined with real-time quantitative PCR. Results are shown in arbitrary units of expression relative to the standard curve obtained from 10-fold dilutions of mouse reference RNA and normalized to the expression of hypoxanthine guanine phosphoribosyl transferase 1 (HPRT). Data are shown as mean ± SEM.

associated receptors, in this case VIP-treatment, may not augment IFN-γ levels. Since specific $VPAC_2$-R agonists were unavailable in our study, the contribution of these receptors to the pathogenesis of the disease remains to be investigated.

Contrary to previous reports [7], systemic immune parameters (e.g. levels of circulating cytokines and spleen lymphocyte populations) were not affected by the VIP or ($Ala^{11,22,28}$)-VIP treatments in $ApoE^{-/-}$ mice. Apparently, systemic effects of the treatments may differ depending on the disease model. As both $VPAC_1$-R and $VPAC_2$-R are expressed in the lesions, it is more likely that the observed effects are elicited locally.

Our study has important limitations. Circulating ($Ala^{11,22,28}$)-VIP concentrations could not be measured since the anti-VIP antibodies used in the radioimmunoassay did not cross-react with ($Ala^{11,22,28}$)-VIP. However, clear changes in atherosclerosis burden in ($Ala^{11,22,28}$)-VIP-infused mice confirm that this compound was delivered properly.

In our experiments, we used relatively young $ApoE^{-/-}$ mice. At the age of 12 weeks, when the animals were implanted with minipumps, the former are practically free of lesions, except for subendothelial collections of foam cells and small groups of foam cells in the aortic root [31]. It will be of interest to determine whether VIP

and ($Ala^{11,22,28}$)-VIP treatments affect established atherosclerosis as well.

In summary, our study shows that in the $ApoE^{-/-}$ mouse model of early atherosclerosis, infusion of a specific $VPAC_1$-R agonist leads to an increase in lesion size with concomitant immune activation of the lesions. Administration of VIP does not alter the course of the disease. This first report of VPAC-R involvement in atherosclerosis development could instigate further dissection of the underlying biology and pharmacology of VPAC receptors in this disease.

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