Stress-induced modulation of the innate immune system in cardiovascular disease

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Chapter 6

Neuropeptide Y receptor Y1, Y2 and Y5 antagonism accelerates atherosclerotic lesion development in LDL receptor-deficient mice

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Abstract

Objective: Neuropeptide Y is an abundantly expressed stress–related hormone capable of modulating metabolic and immune responses involved in the development and progression of atherosclerosis. NPY mediates its effects via multiple G-protein coupled receptors (Y1-Y6), which are differentially expressed throughout the central nervous system and the periphery. Here we investigated the therapeutic potential of systemic NPY Y1, Y2 or Y5 receptor antagonism on atherosclerosis development in $LDLr^{-/-}$ mice.

Methods and Results: Gene expression profiles of NPY and its receptors were obtained by qPCR analysis of collar-induced atherosclerosis specimens and guide wire-induced restenotic carotid arteries. NPY was significantly increased during disease progression in both models, while Y1 receptor expression was almost completely abolished. Expression of Y2 and Y5 as well as DPPIV was generally increased, rendering intervention in Y2 and Y5 signalling of therapeutic interest. Systemic treatment of high fat diet-fed $LDLr^{-/-}$ mice with the Y1 (BIBO-3304), Y2 (BIIE-0246) or the Y5 (CGP-71683) antagonists for 6 weeks resulted in increased lesion development, especially in the Y2 receptor antagonist treated mice. Changes in triglyceride metabolism in white adipose tissue and a general increase in systemic inflammation, e.g. the pro-inflammatory and proatherogenic IL-12 family cytokines, seem to contribute to the observed increase in atherosclerosis.

Conclusions: NPY and its receptor system is highly involved in a wide array of physiological and pathological processes including atherosclerosis. Here we provide evidence for a pro-atherogenic effect of chronic systemic NPY receptor antagonism acting through both metabolic changes and inflammatory cytokine production.

Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide, is a ubiquitous hormone with well-established central and peripheral functions, ranging from regulation of energy balance to modulation of immune responses. NPY is expressed in the brain and peripheral nervous system and acts through multiple G-protein coupled receptors (Y1-Y6), which are differentially expressed throughout the body.[1]

After its discovery by Tatemoto *et al*. in 1982[2], NPY-like immunoreactivity was demonstrated in specific areas of the brain, especially the hypothalamic brain structures, and peripherally throughout the sympathetic nervous system. Peripheral NPY distribution parallels that of tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase, both rate limiting enzymes in the catecholamine synthesis. NPY is often co-stored and co-released with norepinephrine and dopamine. High concentrations of NPY were demonstrated in highly innervated tissues, such as the heart, spleen, kidney and around blood vessels. The connection with the stress response is further strengthened by bidirectional modulation of the expression of NPY by glucocorticoids and vice versa.[3]

The first identified biological function of NPY was its potent vasoconstrictive effect on multiple vascular beds, including the cerebral and renal arteries[4,5,6] and points towards involvement of NPY signalling in vascular homeostasis and disease. Extensive work by Zukowska et al. elucidated potent growth promoting properties of NPY on vascular smooth muscle cells, endothelial cells and adipocytes. Signalling through its Y1 or Y2 receptor was demonstrated to induce vascular smooth muscle cell and endothelial cell proliferation contributing to angiogenesis.[7,8] Furthermore, the NPY-NPY2R system was shown to be involved in stress-induced augmentation of obesity and metabolic syndrome by stimulation adipogenesis, as well as white adipose tissue inflammation and angiogenesis.[9]

While all animals express NPY and its receptors, clear species differences exist. For example, the Y4 receptor is found in rodents and has high affinity for the NPY-related peptide PP but is not found in humans. The Y6 receptor is expressed in many species except the rat and only in a truncated form in various primates, including humans.[10] In addition, the different receptors have different binding preferences for NPY-family members and truncated forms of the NPY protein. A key enzyme involved in the proteolysis of NPY is the abundantly expressed dipeptidyl peptidase IV (DPPIV) cleaving NPY into NPY3-36, which lacks Y1 receptor affinity and thus shifts the responses towards Y2- and Y5 receptor mediated effects. High vascular expression of NPY is considered to be pro-atherogenic. A gain-of-function mutation in the preproNPY gene strongly correlates with increase intima media thickness[11] and increased expression of NPY and the Y1, Y2 and Y5 receptors was demonstrated in patients with peripheral artery disease in the carotid or femoral arteries by means of immunohistochemical stainings.[12] In addition, NPY signalling

was shown to be intimately linked to stress induced accelerated atherosclerosis in various animal models.[12,13,14] Furthermore, we recently indicated plaque NPY to be associated with plaque vulnerability and NPY-induced mast cell activation as a contributing mechanism to atherosclerosis development.[15] Like mast cells, many, if not all, cells of the immune system express functional NPY receptors. For example, NPY induced polarization of T cell responses[16] and pro-inflammatory cytokine production by macrophages[17] are likely mechanisms involved in NPY-mediated accelerated atherosclerosis.

With respect to NPY receptor antagonism in atherosclerosis, the main focus has been on the Y1 receptor-mediated vasoconstrictive and growth promoting properties, mostly in concert with Y5. Accelerated angioplasty-induced restenosis by local NPY treatment was shown to be completely blocked continuous infusion with H409/22 or CGP71683, which are Y1 or Y5 receptor antagonists, respectively.[13] Similar beneficial results were obtained in a restenosis model in apoE$^{-/-}$ mice by BIBP-3226 treatment, another Y1 receptor specific antagonist.[18] In contrast, a recent study evaluating systemic Y1 receptor antagonism in apoE$^{-/-}$ mice fed a high fat diet, a model of atherosclerosis rather than restenosis, surprisingly demonstrated accelerated atherosclerosis in NPY and Y1 receptor antagonist treated mice.[19] Mainly pro-atherogenic inflammatory IL-12 production and possibly increased leptin secretion was thought to result in increased atherosclerosis under hyperlipidemic conditions.

Considering the proatherogenic effects of NPY, and the vascular expression of the different NPY receptors and differential expression at specific stages of human disease progression[20], we aimed to determine NPY receptor expression during the development and progression of atherosclerosis and subsequently evaluate the therapeutic potential of systemic Y1, Y2 or Y5 receptor antagonism in the prevention of atherosclerosis.

Materials and Methods

Neuropeptide Y and receptor expression during atherosclerosis development and restenosis

All animal studies were performed in compliance with Dutch government guidelines, the Directive 2010/63/EU of the European Parliament and were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 12102). Diet and water were provided ad libitum.

To determine the gene expression profiles of NPY, Y1R, Y1R, Y1R and DPPIV during atherosclerotic lesion progression apoE$^{-/-}$ mice were put on Western type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK) and equipped with bilateral semi-constrictive collars (2mm long, 0,3mm in diameter) around the left and right common carotid artery as described by von der Thusen *et*

al.[21] Before and at 2 week intervals after collar placement, 6 mice were sacrificed and the carotids isolated for gene expression analysis. Total RNA was extracted from 3 pooled carotids (n=4 per timepoint) with the guanidium thiocyanate-phenol-bromochloropropane extraction method[22] and RNA concentration, purity and integrity were examined by nanodrop (Nanodrop® Technologies). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Landsmeer, The Netherlands) and used for quantitative PCR analysis with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems). Murine HPRT, RPL27, Gusb and 36B4 were used as standard housekeeping genes. qPCR primer pairs are given in supplemental table 1. A similar setup was used to determine the gene expression of NPY and its receptors during progression of restenosis using a wire-induced denudation model as described previously.[23,24] In short, the right carotid artery was dissected free and denudated using a 0.36-mm guide wire, which results in a proliferative VSMC-rich lesions within 4 weeks. Before and every week after denudation 6 mice were sacrificed and the carotids isolated for gene expression analysis.

Systemic NPY receptor (Y1, Y2 or Y5) antagonism during atherosclerosis development

To study the effect of NPY receptor antagonism on atherosclerosis development we fed low-density lipoprotein receptor deficient ($LDLr^{-/-}$) mice a Western-type diet (WTD) for eight weeks. Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands) and treatment groups were randomized based on age and weight at the start of the experiment. After 2 weeks on WTD and onwards mice were injected i.p. three times a week with 100ul PBS (1% DMSO), Y1 receptor antagonist BIBO-3304 trifluoroacetate (400μM; #2412; Tocris Bioscience, Ellisville, MO, USA), Y2 receptor antagonist BIIE-0246 (350μM; #1700; Tocris Bioscience) or Y5 receptor antagonist CGP 71683 hydrochloride (750μM; #2199; Tocris Bioscience). Bodyweight, serum cholesterol and triglyceride levels were checked regularly throughout the study. Hematological parameters (including white blood cell count and leukocyte subpopulations) were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). After 6 weeks of treatment, mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 and 2 mg/kg, respectively, Janssen Animal Health, Sauderton, UK). Adequacy of anaesthesia was monitored by regular visual inspection and toe pinch reflex. Mice were exsanguinated via orbital bleeding and in situ perfused with PBS after which the hearts were excised and stored in 3.7% neutral-buffered formalin (Formal-Fixx, Shandon Scientific Ltd., Runcorn, UK) for further analysis.

Histological analysis and morphometry

To determine plaque size, serial 10 µm cryosections of the aortic root were cut using a Leica CM3050S cryostat and stained with Oil-Red-O and hematoxylin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Plaque size was analyzed in 5 consecutive sections, starting at the point where all three aortic valve leaflets first appeared, with a Leica DM-RE microscope and LeicaQwin software and represented as mean plaque area and total plaque volume (AUC). Corresponding sections on separate slides were stained with a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, Serotec, Raleigh, NC, US) and alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich). A Masson's trichrome (Sigma-Aldrich) staining was performed to analyze plaque collagen content and necrotic core area, which was defined as the a-cellular, debris-rich plaque area. Macrophage, collagen and necrotic core area were measured as percentage of total plaque area. Medial and intimal vascular smooth muscle cell content was determined by α-smooth muscle cell actin staining (Sigma-Aldrich). Mast cells were visualized with a naphthol AS-D chloroacetate esterase staining kit (Sigma-Aldrich) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. All morphometric analyses were performed by blinded independent operators.

Spleen and liver RNA isolation and gene expression

At sacrifice spleen and liver tissue were isolated, immediately snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. After tissue homogenization with a Potter tissue homogenizer Total RNA was extracted, reverse transcribed and used for gene expression analysis as described above.

Serum cholesterol and triglyceride levels

Serum concentrations of total cholesterol and triglyceride were determined by enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany) in 96-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Precipath (standardized serum, Roche Diagnostics) was used as internal standard in the cholesterol and triglyceride assay.

Statistical analysis

Data are expressed as mean ± SEM. An unpaired two-tailed Student's t-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two groups with more than one variable were analyzed by two-way ANOVA, both followed by Tukey's multiple comparison test. A level of $P<0.05$ was considered significant.

Results

Differential expression of NPY and its receptors during atherosclerosis and restenosis

To investigate the involvement of NPY signaling through its G-protein coupled receptors Y1, Y2 or Y5, in atherosclerotic lesion development we first determined gene expression levels in two different vascular disease models (i.e. a collar-induced atherosclerosis model and a wire-induced denudation model of restenosis). Atherosclerotic lesions were induced in apoE$^{-/-}$ mice fed a Western-type diet by means of bilateral collar placement. Similar to previously published microarray results[15], expression of NPY was significantly increased during atherosclerotic lesion development (figure 1A). Interestingly, while Npy1r was highly expressed in healthy carotid arteries, its expression was almost completely abolished upon disease initiation and remained low during the whole experiment. Expression of Npy2r and Npy5r fluctuated but was generally higher in advanced atherosclerosis. Interestingly, DPPIV expression was highly induced and mirrored the effect on expression of the Y1 receptor, possibly further inducing NPY signaling via the Y2 and Y5 receptors. As NPY is known to have potent vasoconstrictive and angiogenic properties, we analyzed the expression NPY and its receptors in restenotic vessels, which is a primarily VSMC proliferation-driven process, in contrast to atherosclerosis, which is known to mainly be macrophage- and lipid-driven. Strikingly, the expression profiles were very similar to the atherosclerotic lesions, with increased NPY expression especially at later stages of the disease and a severe reduction in Npy1r expression (figure 1B). In contrast to the atherosclerotic lesions, Npy2r and Npy5r expression was initially reduced after endothelial denudation, but recovered gradually to baseline expression levels at 4 weeks.

Effects of systemic NPY receptor antagonism on bodyweight, cholesterol and triglyceride homeostasis and glucocorticoid levels.

The differential expression of the NPY receptors on various tissues and cell types involved in atherosclerosis combined with the expression profiles during atherosclerosis development prompted us to investigate the effects of Y1, Y2 and Y5 receptor antagonists in an atherosclerotic mouse model. To induce atherosclerosis we fed male LDL$^{r-/-}$ mice a high cholesterol diet for 8 weeks combined with systemic antagonist treatment for the Y1, Y2 or Y5 receptor. As central NPY is a potent orexigenic hormone, mediating appetite, hepatic triglyceride (TG) secretion and TG storage in white adipose tissue[25,26], we determined bodyweight, total cholesterol and triglyceride levels throughout the study. No apparent effect of the antagonist on bodyweight or serum cholesterol could be observed (figure 2A; B), however plasma triglyceride levels were significantly elevated at 4 weeks

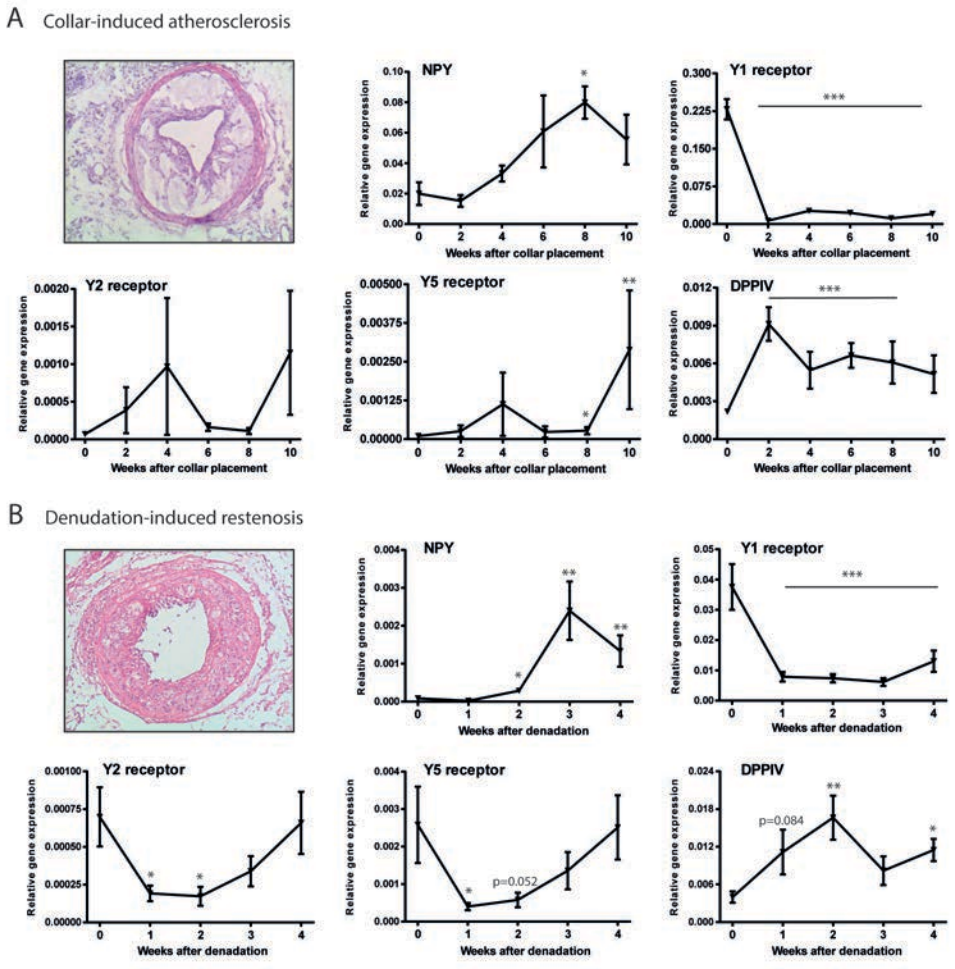

Figure 1. Gene expression levels of NPY, its receptors Y1, Y2, Y5 and peptidase DPPIV the during (A) carotid artery atherosclerosis progression induced by semi-constrictive collar placement and (B) carotid guide wire-induced restenosis.

of Y1 treatment (figure 2C). As previous research indicated the effect of central NPY infusion on TG levels to act within 2 hours, we determined plasma TG levels 1,5 hour after injection with the antagonists. A significant increase in plasma TG levels upon Y1 and Y5, but not Y2 receptor antagonism could be observed (figure 2D). NPY is co-released with catecholamine and glucocorticoids during stress and NPY receptor expression is abundant on the adrenal glands.[27] To account for the possible contribution of changes in circadian levels of glucocorticoids, we measured morning and late afternoon plasma corticosterone levels after 5 weeks of receptor antagonist treatment. As depicted in figure 2E, no significant differences in circulating hormone levels could be observed.

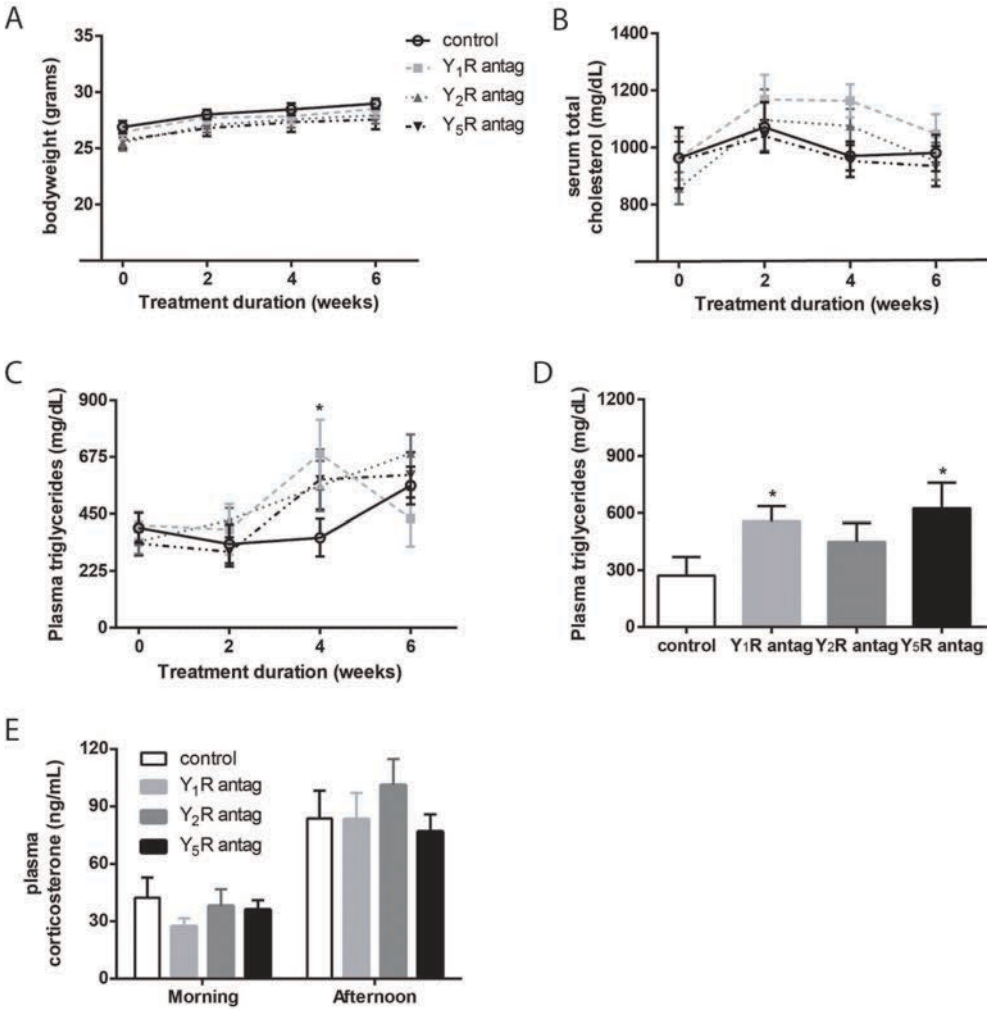

Figure 2. A) Bodyweight throughout the experiment. Mice were put on Western type diet t = -2 weeks before the start of NPYr antagonist treatment at t = 0. B) Total serum cholesterol levels at 2 week intervals during the treatment period. C) Plasma triglyceride levels at two week intervals during the antagonist treatment period. Significant increase in TG levels at 4 weeks of Y1 receptor antagonist treatment compared to control. D) Plasma triglyceride levels 1,5 hour after antagonist injection at 5 weeks of treatment. E) Plasma corticosterone levels in the resting (early morning) and active phase (late afternoon). P <0.05 vs control treatment is considered significant.

Chronic NPY receptor antagonism during atherosclerotic lesion progression.

In our study we monitored circulation levels of the main leukocyte subpopulations (i.e. neutrophils, lymphocytes, monocytes and basophils) during systemic antagonist treatment. We observed limited effects on these cell populations (supplemental figure 1), only Y5 antagonism reduced neutrophil monocyte numbers at two weeks of treatment and Y2 antagonism significantly increase the neutrophil count at sacrifice, after 6 weeks of treatment.

Next we analyzed the atherosclerotic plaque burden in the aortic root by means

of Oil-red-O staining of histological sections. While all antagonist-treated mice appeared to have a higher plaque burden compared to control (1% DMSO) mice, only the Y2 treated mice had statistically significant larger plaques (figure 3A; 1.4-fold increase). In addition to plaque burden, lesion stability or vulnerability to erosion or rupture is an important measure of disease progression and strongly correlates with clinical outcome. We determined lesion stability by quantifying the macrophage and collagen content of the plaque, as well as and the necrotic core area. The amount of intimal macrophages (as percentage of total plaque area) was significantly reduced in Y1 and Y2 antagonist treated mice (Figure 3B).

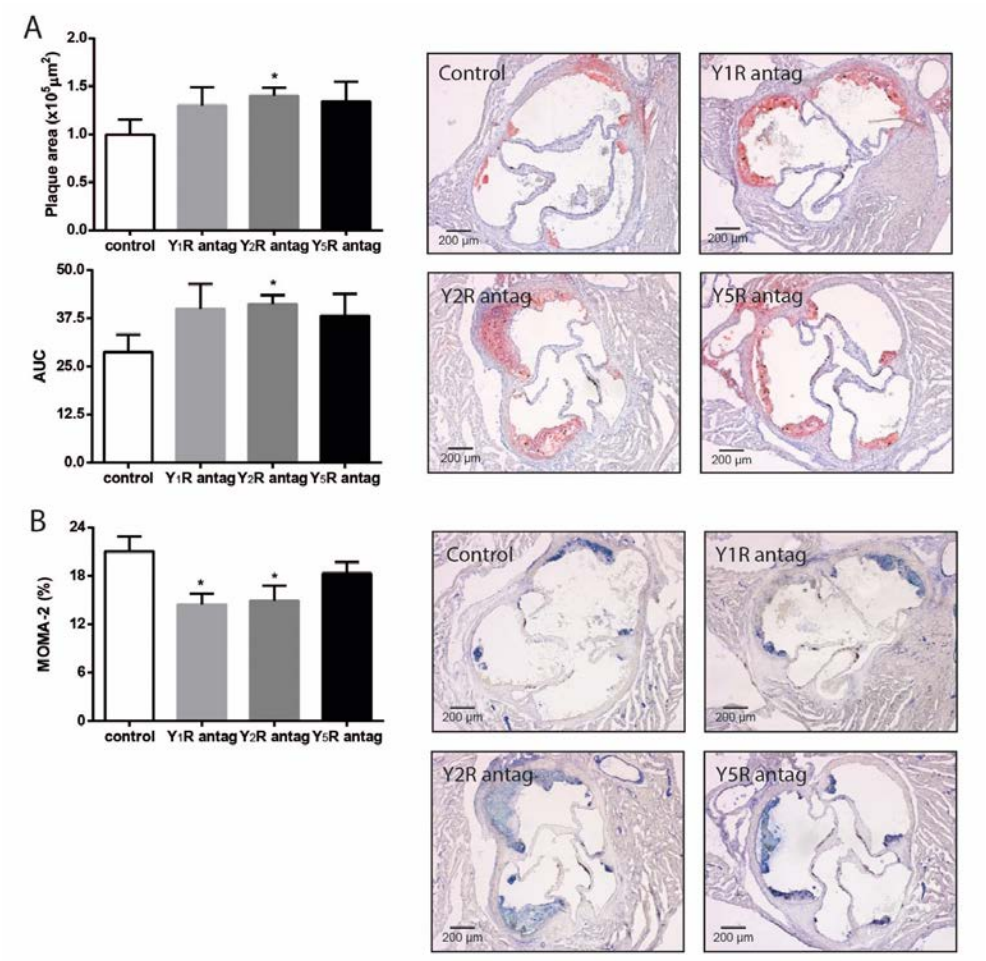

Figure 3. A) Atherosclerotic lesion formation in the three valve area of the aortic root was assessed by Oil-red-O staining. B) Macrophage content of the lesions as determined by MOMA-2 staining and depicted as percentage of lesion area.

As most lesions were early lesions, intimal collagen deposition was limited and not significantly different between treatment groups (figure 4A). Also the amount of necrosis was not significantly different, although appeared somewhat more

prominent in the larger lesions of Y2 antagonist treated mice. Intimal smooth muscle cells, measured by α-smooth muscle staining, were decreased by Y2 antagonist treatment (2.41 ± 0.006% vs 4.43 ± 0.007% in control mice) and demonstrated a trend towards a decrease in the media of Y2 and Y5 antagonist treated mice (figure 4B). Combined, the morphological analysis at least partly confirm the pro-atherogenic effect of systemic Y1 receptor antagonism, but provides additional evidence for an even more pronounced pro-atherogenic role for Y2 receptor antagonist treatment.

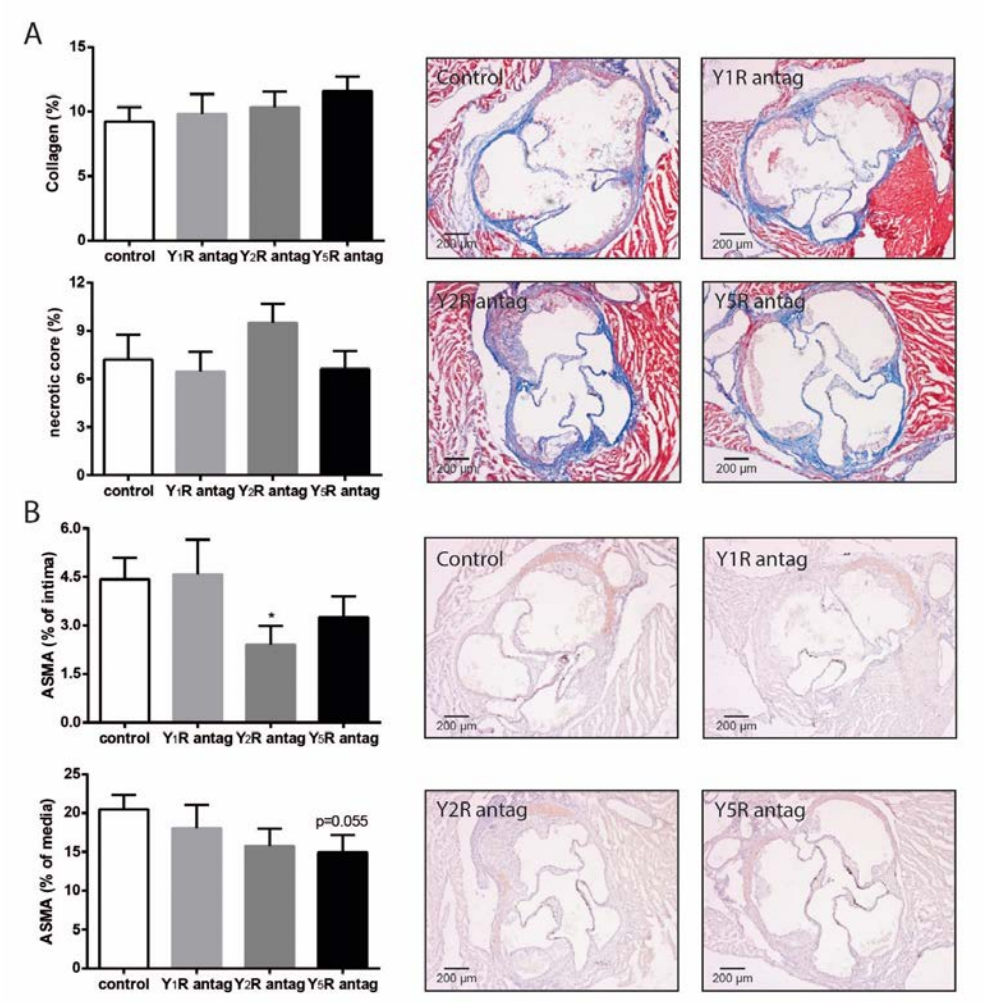

Figure 4. A) Collagen content of the atherosclerotic lesions in the three valve area of the aortic root was assessed by Masson's trichrome staining as percentage of total lesion area. B) Intima and media vascular smooth muscle was determined by alpha smooth muscle actin staining and represented as percentage of the intima or media area.

Mast cell accumulation upon systemic Y2 receptor antagonist treatment.

Previous results from our lab have implicated NPY-induced perivascular mast cell activation as a potential contributing factor to atherosclerotic lesion development.[10] To evaluate the effect of systemic NPY receptor antagonist treatment on perivascular mast cell responses we quantified the amount of cardiac mast cells in the aortic root sections and determined their activation status. A trend towards more mast cells in the Y2 antagonist treated mice (p=0.055) could be observed (figure 5A). However, the percentage of activated mast cells was similar between treatment groups (figure 5B), suggesting the increased number to primarily reflect the more advanced and inflamed plaque in the Y2 antagonist mice.

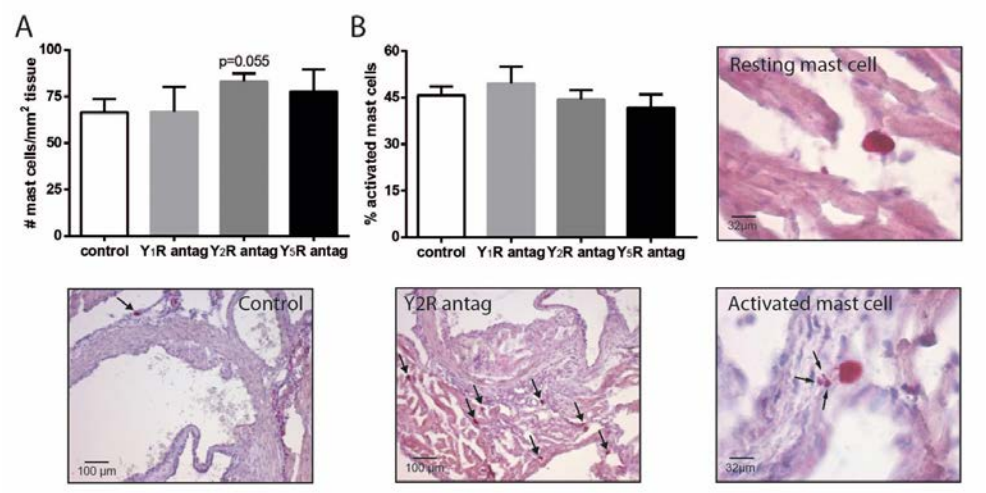

Figure 5. A) Mast cells were visualized and scored by naphtol AS-chloroacetate staining. B) Activation status is illustrated by the presence of granules in the surrounding tissue.

NPY receptor antagonist induced changes in the pro-atherogenic IL-12 cytokine family.

Earlier data on systemic Y1 receptor treatment indicated an important role for the pro-inflammatory cytokine IL-12 in mediating the increase in atherosclerosis.[19] To evaluate the contribution of the IL-12 cytokine family, we determined the expression levels of p40, p35, p19, p28 and Ebi3 in the liver and spleen. The IL-12 subunits p40 and p35 were not significantly altered by antagonist treatment, however we did observe a significant reduction in p35 expression in the liver after Y5 antagonist treatment. The observed changes in gene expression in correlated to some extent with the circulating levels of IL-12 at sacrifice, demonstrating higher levels in the Y1 and Y2 treated mice and a reduction upon Y5 antagonist treatment (Suppl fig. 2A; top left panel). Interestingly, expression of Ebi3, which in complex with p28 forms the proatherogenic cytokine IL-27[29], was significantly higher in the Y1 treated mice and trended towards higher expression in the Y5

mice. Liver expression levels of Ebi3 were similarly increased in Y1 antagonist treated mice, but reduced in Y5 treated mice. (supplemental figure. 2B)

Discussion

Atherosclerosis and its clinical manifestations remain a leading cause of death and an enormous burden on society. Both metabolic- and immune-dysregulation are key pathological processes involved in atherosclerotic lesion development and progression. In addition, increased recognition is given to the brain-immune and brain-gut axis in contributing to various diseases, including cardiovascular disease. Neuropeptide Y is an abundant (stress-related) hormone with variety of known functions both in health and disease. Being co-released with and potentiating the effects of norepinephrine and ATP released from sympathetic neurons innervating for instance the vasculature, NPY has been shown to be pro-atherogenic and responsible for mediating stress-induced accelerated restenosis and atherosclerosis.[30]

In the current study we investigated the therapeutic potential of systemic NPY receptor antagonism of each of its three main receptors, Y1, Y2 and Y5 in atherosclerosis. First, expression of NPY and its receptors was assessed in atherosclerotic and restenotic carotid artery specimens. As previously reported by us and others, NPY expression was significantly increased in atherosclerotic arteries. Similar to expression levels observed in human carotid endarterectomy specimens[20], Npy1r receptor expression was significantly reduced upon disease initiation, which possibly questions the usefulness of Y1 receptor antagonism. However, contrasting data demonstrating increased expression of all three NPY receptors in the carotids and femoral arteries of patients with peripheral artery disease compared to healthy iliac arteries also exist.[12] In our study, Npy2r and Npy5r expression was generally increased and combined with the strong induction of DPPIV may suggest a shift towards Y2 and Y5-mediated effects.

Morphological analysis of the plaque burden in the aortic root after 6 weeks of NPY receptor antagonist treatment revealed a significant increase in plaque size in the Y2 receptor antagonist treated mice. In fact, also Y1 and Y5 receptor antagonism showed a trend towards more advanced lesions. Although the amount of circulating monocytes, or any of the other main white blood cell subpopulations, was not significantly altered during the treatment period, plaque macrophage content was significantly decreased by Y1 and Y2 antagonist treatment. Whether this is a direct effect on macrophage function or on monocyte recruitment via changes in endothelial adhesion molecule expression requires addition research. In vitro neutrophil and monocyte adhesion to human umbilical vein endothelial cells was previously shown to be significantly increased upon incubation with NPY[31] and NPY has been shown to have a variety of stimulatory and inhibitory functions on

macrophages and T cells via its different receptors.[16, 17]

We and others previously observed increased NPY expression in vulnerable atherosclerotic lesions compared with stable lesions, suggesting the involvement of NPY in lesion destabilization.[15,20] In line with previous results obtained with local Y1 receptor antagonism in an endothelial denudation model[18], systemic administration of NPY receptor antagonists did not result in a significant difference in collagen content nor a difference in necrotic core area of the plaques between the treatment groups. However, lesions were relatively small and primarily foam cell-rich lesions, which generally lack a clear collagen rich cap or intimal necrosis. Furthermore, no extensive differences in VSMC content of the lesions was observed except for a significant decrease in intimal α-SMA staining in the Y2 antagonist treated mice and a trend towards a decrease in the Y5 receptor antagonist treated mice. Previous results from restenosis models actually observed reduced intimal hyperplasia upon antagonism of the Y1 or Y5 receptor or both receptors combined[13,18], resulting of reduced smooth muscle cell proliferation. Lack of such effects in our atherosclerosis model may be reflect the strongly reduced Y1 receptor expression and relative initial, and thus smooth muscle cell poor, lesions. Perivascular mast cell activation and IL-6 secretion was previously shown to contribute to NPY-induced accelerated lesion development in apoE$^{-/-}$ mice[15], suggesting the potential anti-inflammatory benefit of NPY receptor antagonism. However, in line with an overall increase in atherosclerosis progression and inflammation in the Y2 receptor antagonist treated mice, mast cell numbers were actually increased in the cardiac tissue surrounding the aortic root without significant differences in activation status. Interestingly, plasma triglyceride levels were transiently increased at 4 weeks of Y1 receptor antagonist treatment and especially elevated shortly (1,5h) after both Y1 and Y5 antagonist injection. Previous results on acute central NPY infusion demonstrated increased food intake and triglyceride secretion in rats mediated via the Y1 receptor.[32] In mice however, NPY administration increased food intake without affecting hepatic VLDL-TG production. Whether the observed increase in TG secretion in our model reflects central modifications or peripheral effects deserves further investigation.

In agreement with previous results demonstrating an increased systemic inflammatory response upon Y1 receptor antagonism, splenic and liver expression of subunits of the pro-inflammatory cytokine IL-12 family were increased in the Y1 and Y5 treated mice and somewhat reflected in increased circulating level of IL-12 p70. Interestingly, especially Ebi3, which in complex with p28 form the atherogenic cytokine IL-27, was significantly increased by systemic Y1 receptor antagonism, suggesting the potential proatherogenic effect of Y1 antagonism to be mediated by multiple IL-12 family members. Of note, Y2 receptor antagonism did not affect the IL-12 cytokine family similarly, suggesting other mechanisms to account for accelerated atherosclerosis in those mice. The limited data on the

effects of Y2 signaling points toward its involvement in monocyte migration[33] and angiogenesis[34], which do not necessarily explain the increase in atherosclerosis observed here. Interestingly, recent clinical trial data of DPPIV antagonist use for glycemic control in T2DM patients indicated increased heart failure upon long-term use.[35] While NPY signaling was not evaluated, reduced signaling via the Y2 receptor may be a contributing factor.

In conclusion, our data provides further insight in the intricate role of NPY and its widely expressed receptor system in atherosclerosis development and progression. In contrast to the beneficial effects local application of Y1 receptor antagonists at sites or restenosis, systemic Y1, Y2 and Y5 receptor antagonism in a hyperlipidemic atherosclerosis model resulted in increased lesion development. Here, especially Y2 receptor antagonism increased lesion progression resulting in a more advanced state of the lesions in those mice. In light of recent detrimental cardiovascular side effects of long term DPPIV inhibitor treatment in T2DM patients[35,36], systemic modulation of the NPY-receptor system should be approached with caution and further research especially into pathological mechanisms of Y2 receptor signaling is warranted.

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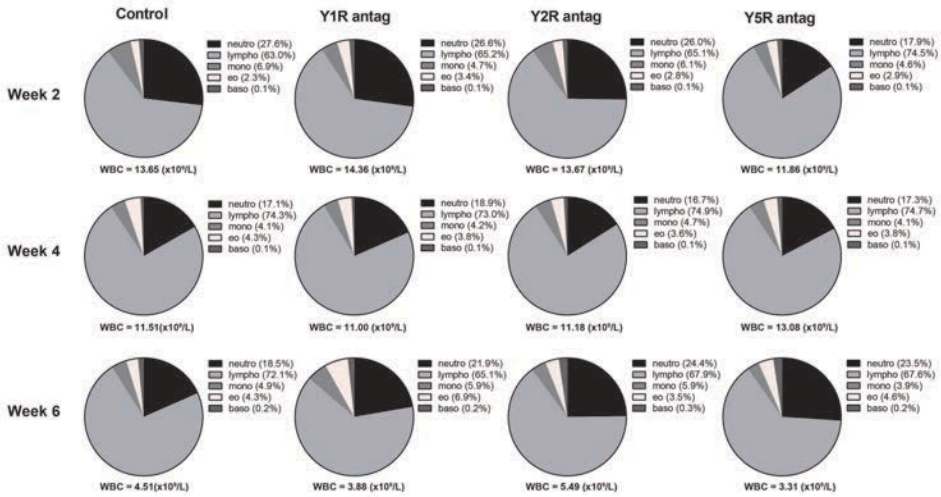
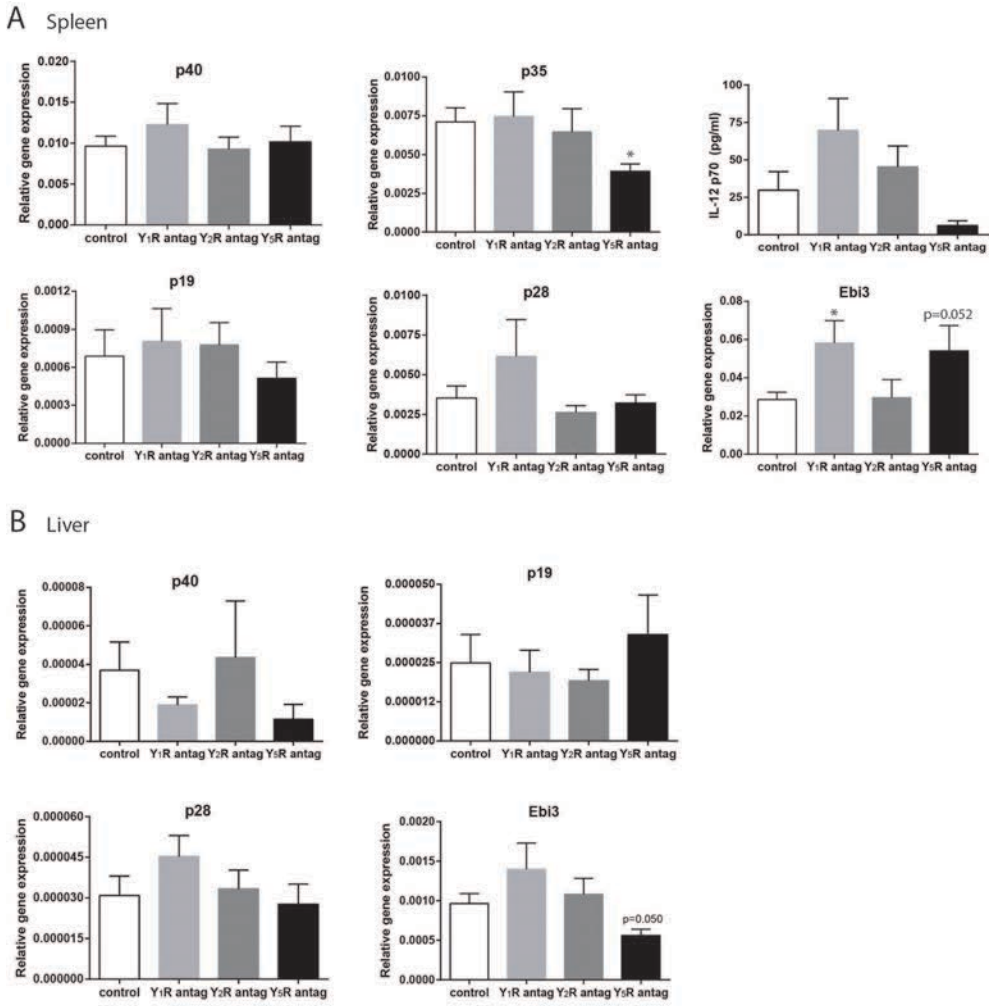
Supplemental data

Supplemental table 1: qPCR primers

Gene	Forward (5'-3')	Reverse (5'-3')
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
Gusb	TATGGAGCAGACGCAATCCCAG	AGCTCTCCGACCACGTATTCTTTAC
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
NPY	ACATCAATCTCATCACCAGACAG	AGTTTCATTTCCCATCACCACA
NPY1R	ACACTCGTCCCGCTTCAACA	TCTTCAAAACGGATCAAATCTTCAGCA
NPY2R	GAAGGAACGCGCAAGAGTCAATAC	CCCATAGGGCTCCACTTTCACTT
NPY5R	GATGCTCAGGAGATGAGAGTCAA	TCCAGCTAACAGCGAACACTAA
p40	GATTCAGACTCCAGGGGACA	GGAGACACCAGCAAAACGAT
p35	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
p19	GCCTGCTCTACTCCCTGATAGC	TGGGCATCTGTTGGGTCT
p28	CACAGGCACCTCCGCTTT	TTGGGATGACACCTGATTGG
Ebi3	CCCGGACATCTTCTCTCTCA	CAATACTTGGCATGGGGTTT



Supplemental figure 1. Relative blood leukocyte composition at two week intervals after the start of antagonist treatment, demonstrating no clear systemic effects on leukocyte subpopulations in blood.



Supplemental figure 2. Gene expression of IL-12 family subunits in A) spleen and B) liver after 6 weeks of systemic NPY receptor antagonist treatment.

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