Melanocortin overexpression limits diet-induced inflammation and atherosclerosis in LDLR$^{-/-}$ mice

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Abstract

Atherosclerosis is a chronic inflammatory disease of the arteries. The disease is initiated by endothelial dysfunction that allows the transport of leukocytes and low-density lipoprotein into the vessel wall forming atherosclerotic plaques. The melanocortin system is an endogenous peptide system that regulates, for example, energy homeostasis and cardiovascular function. Melanocortin treatment with endogenous or synthetic melanocortin peptides reduces body weight, protects the endothelium and alleviates vascular inflammation, but the long-term effects of melanocortin system activation on atheroprogression remain largely unknown. In this study, we evaluated the effects of transgenic melanocortin overexpression in a mouse model of atherosclerosis. Low-density lipoprotein receptor-deficient mice overexpressing alpha- and gamma$_3$-MSH (MSH-OE) and their wild-type littermates were fed either a regular chow or Western-style diet for 16 weeks. During this time, their metabolic parameters were monitored. The aortae were collected for functional analysis and the plaques in the aortic root and arch were characterised by histological and immunohistochemical stainings. The aortic expression of inflammatory mediators was determined by quantitative PCR. We found that transgenic MSH-OE improved glucose tolerance and limited atherosclerotic plaque formation particularly in Western diet-fed mice. In terms of aortic vasoreactivity, MSH-OE blunted alpha$_1$-adrenoceptor-mediated vasoconstriction and enhanced relaxation response to acetylcholine, indicating improved endothelial function. In addition, MSH-OE markedly attenuated Western diet-induced upregulation of proinflammatory cytokines (*Ccl2*, *Ccl5* and *Il6*) that contribute to the pathogenesis of atherosclerosis. These results show that the activation of the melanocortin system improves glucose homeostasis and limits diet-induced vascular inflammation and atherosclerotic plaque formation.

Introduction

The most acute complications of cardiovascular diseases originate from atherosclerosis, a chronic inflammatory disease of the middle- and large-sized arteries (World Health Organization. 2015). One important risk factor for atherosclerosis is the metabolic syndrome, which constitutes abdominal obesity, high cholesterol and high blood pressure as well as diabetes and prediabetes (International Diabetes Federation. 2006). The link between atherosclerosis and impaired glucose homeostasis, a hallmark of diabetes, has been well established in large epidemiological studies (Kannel & McGee. 1979, Turner*, et al.* 1998). In metabolic syndrome, diabetes adds the risk for cardiovascular disease and atherosclerotic complications, as metabolic syndrome with diabetes increases the prevalence of coronary artery disease more than metabolic syndrome without diabetes (Alexander*, et al.* 2003). The metabolic syndrome is often present in type 2 diabetes, but the risk for cardiovascular disease is increased also in type 1 diabetes, where metabolic syndrome is rarer, suggesting that the common features of type 1 and 2 diabetes, such as hyperglycemia, play a major role in the development of cardiovascular disease (Chait & Bornfeldt. 2009).

According to the current understanding, several factors, including hyperglycemia and hyperlipidemia, may cause endothelial dysfunction that is the initiating event of atherosclerosis. Endothelial dysfunction permits the infiltration of immune cells, mainly macrophages and T cells, and low-density lipoprotein (LDL) into the subendothelial space, where they eventually form atherosclerotic plaques and impair the vascular homeostasis (Viola & Soehnlein. 2015).

The majority of current treatment strategies for atherosclerosis are based on lowering blood cholesterol and particularly LDL cholesterol levels, which have proven to be effective for

most patients. Nevertheless, the acute complications in cardiovascular diseases still cause more than 30% of all deaths (Mozaffarian*, et al.* 2015, World Health Organization. 2015), calling for new treatment strategies. The concept of restoring endothelial dysfunction has gained wide interest in the development of anti-atherosclerotic therapies (Khan*, et al.* 2015, Koenen & Weber. 2011) and one such promising target is the melanocortin system.

The melanocortin system consists of the melanocortin peptides, alpha-, beta- and gamma-melanocyte-stimulating hormones (alpha-, beta- and gamma-MSH), and corticotrophin; five melanocortin receptors, named MC1R-MC5R (Mountjoy*, et al.* 1992), and their antagonists, agouti and agouti-related protein (Cortes*, et al.* 2014, Nakanishi*, et al.* 1979). A wealth of evidence has recognised the benefits of the melanocortin system activation on cardiovascular, inflammatory and metabolic regulation both *in vitro* and *in vivo* (Brzoska*, et al.* 2008, Catania*, et al.* 2010, Leoni*, et al.* 2008, Leoni*, et al.* 2010, Patel*, et al.* 2011, Rinne*, et al.* 2013, Rinne*, et al.* 2014, Schaible*, et al.* 2013). Recently, we and others showed that alpha-MSH and its analogue, melanotan 2, evoke anti-inflammatory and vasoactive effects both in endothelial cells and in a mouse model of atherosclerosis (Rinne*, et al.* 2014, Yang*, et al.* 2015). On the other hand, deficient MC1R function disturbs the vascular endothelial function both in mice and humans (Rinne*, et al.* 2015). The vasoprotective effects arise from the augmentation of nitric oxide availability (Davignon & Ganz. 2004, Rinne*, et al.* 2013), whereas the alleviation of inflammation stems from the inhibition of nuclear factor kappa B-driven inflammation (Manna & Aggarwal. 1998, Yang*, et al.* 2015). Melanocortin activation reduces the expression of pro-inflammatory cytokines, their receptors and adhesion molecules (May & Ghosh. 1998) and, on the other hand, induces anti-inflammatory processes (Holloway*, et al.* 2015). Apart from MC1R, MC3R is also instrumental in mediating the anti-inflammatory and vasoprotective effects of melanocortins. Pharmacological treatment with an

MC3R agonist attenuates cell adhesion, emigration and chemokine generation, while deficiency in *Mc3r* leads to increased extravasation and upregulation of proinflammatory markers (Leoni*, et al.* 2008). Moreover, recent studies have shown that alpha-MSH improves glucose uptake to muscle, which alleviates the detrimental effects of hyperglycemia on the vasculature (Breit*, et al.* 2016, Enriori*, et al.* 2016, Moller*, et al.* 2016). Glucose homeostasis is also improved by transgenic alpha- and gamma$_3$-MSH overexpression (MSH-OE) in lean mice as well as in genetic and diet-induced obesity (Lee*, et al.* 2007, Savontaus*, et al.* 2004).

Although the beneficial effects of the melanocortins on inflammation, cardiovascular and metabolic regulation have been clearly characterised, the long-term effects of melanocortin activation on atherosclerosis remain unclear. To this end, we generated an atherosclerotic low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mouse model that overexpresses alpha- and gamma$_3$-MSH and characterised its vascular and metabolic phenotype. Here we report that transgenic MSH-OE improves glucose tolerance and limits the plaque accumulation and the progression of vascular inflammation in *Ldlr*$^{-/-}$ mice.

Materials and methods

Animals

All animal experiments were approved by the Animal Experiment Board in Finland (license number ESAVI-438/04.10.03/2012) and conducted according to European Union Directive 2010/63/EU. Animals were housed on a 12 h light/dark cycle and had free access to water and food.

Previously generated transgenic mouse model overexpressing alpha- and gamma$_3$-MSH was crossbred with mice deficient in low-density lipoprotein receptor (LDLR). The transgene encodes *N*-terminal pro-opiomelanocortin, including alpha- and gamma$_3$-MSH, and is under the control of the cytomegalovirus promoter that drives the expression of *N*-terminal pro-opiomelanocortin in all tissues. Melanocortin peptide levels are increased twofold in the tissues where pro-opiomelanocortin is normally processed to active MSH peptides (Savontaus*, et al.* 2004).

All experiments were performed with transgenic female (n = 10) and male (n = 19) MSH-OE-*Ldlr*$^{-/-}$ (MSH-OE) mice and their *Ldlr*$^{-/-}$ female (n = 11) and male (n = 18) wild-type (WT) littermates. At the age of 3 months, male mice were randomly assigned to two diet groups; regular chow diet (certified reference material; CRM) or high-fat and –sugar Western diet (D12079B, Research Diets Inc.). All female mice were placed on the Western diet. The energy content of the Western diet was 4.7 kcal/g and it composed of 17 kcal% protein, 43 kcal% carbohydrate, and 41 kcal% fat, where 0.21 kcal% came from cholesterol. The chow diet (product code #801722, CRM (P), SDS, Essex, UK) contained 3.6 kcal/g and it composed of 22 kcal% protein, 69 kcal% carbohydrate, and 9 kcal% fat. After 4 months of

diet intervention, mice were euthanized via CO_2 asphyxiation and the tissues were collected for further analysis. The study design is presented in Supplementary Figure 1.

Metabolic studies

During the diet-intervention, the body weight was monitored weekly. Body composition was determined prior to the initiation of the diet, and after 2 and 4 months on the diet by quantitative nuclear magnetic resonance (NMR) scanning (EchoMRI-700, Echo Medical Systems). Glucose tolerance test was carried out after 3 months on the diet. Mice were fasted for 4 hours and 1 g/kg glucose was administered i.p. Blood samples were withdrawn from tail vein before and 20, 40, 60 and 90 minutes after the glucose injection (Precision Xtra, Abbot Diabetes Care, Abbot Park, IL, USA). After sacrifice, blood was withdrawn from vena cava and serum cholesterol was measured using a fluorometric assay kit (Item no. 10007640, Cayman Chemical). Serum leptin level was determined using an ELISA assay (Item no. EZML-82K, Millipore).

En face Sudan IV staining

The adventitia around the aortic arch was removed and the aortic arch was dissected. Aortic arch samples were fixed in 10% formalin for 24 hours and stored in PBS at 4°C until further use. The fixed aortic arch was dissected longitudinally open from the heart to the left subclavian artery and pinned flat intima upward. For atherosclerotic plaque quantification, *en face* preparations of the aortic arch were stained with Sudan IV (Sigma-Aldrich). 70% (v/v) ethanol was added to the dish for 5 minutes. Filtered 0.5% (wt/vol) Sudan IV solution dissolved equally in 70% (v/v) ethanol and acetone was applied for 6 minutes. Samples were destained with 80% (v/v) ethanol for 3 minutes and then washed with PBS. The stained aorta was mounted on a glass plate under a coverslip using PBS. For quantitative analysis, images

of the stained aorta were captured using Zeiss Stemi 2000-C stereomicroscope and PixeLINK Capture OEM software. The intimal area was limited using image manipulation programme (GIMP 2.8, GNU Image Manipulation Program) and the atherosclerotic plaque area of the total intimal area was determined using automated image analysis software (ImageJ, Fiji, National Institutes of Health, Bethesda, Maryland, USA) with colour deconvolution plug-in.

Histological and immunohistochemical stainings

The aortic roots of male mice were embedded in Tissue-Tek O.C.T. compound (Tissue-Tek®, Sakura Finetek USA Inc, Torrance, CA, USA), frozen in isopentane on dry ice and stored at -70°C until further use. Transverse sections of the aortic root (8 µm) were cut and stained with Oil Red O, Masson's Trichrome, and Mac3 and iNOS (Abcam, Cambridge, UK) primary antibodies for the evaluation of lipid accumulation, collagen deposition, macrophage density and macrophage polarisation, respectively, as described previously (Rinne*, et al.* 2014). For liver histology, a transverse piece of the left lobe was embedded in O.C.T compound (Tissue-Tek®) for cryosectioning. Liver sections were therafter stained with Oil Red O. The stained sections were scanned using Pannoramic 250 digital slide scanner (3DHISTECH Ltd.) and quantified (4 sections/slide/mouse) using image analysis software (ImageJ, Fiji, National Institutes of Health, Bethesda, Maryland, USA) as previously described (Rinne*, et al.* 2014, Rinne*, et al.* 2017).

Hepatic lipid analysis

Liver (100 mg) was homogenized in 500 µl of PBS with 0.1% NP-40 using TissueLyser and then centrifuged to remove insoluble material. Triglycerides were quantified in the liver homogenates using triglyceride determination kit (TR0100, Sigma-Aldrich) according to manufacturer's instructions (Rinne*, et al.* 2017).

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the thoracic aorta and liver of male mice by phenol/guanidine-based extraction (QIAzol Lysis Reagent, Qiagen). RNA quality and concentration were measured by spectrophotometer (BioSpec-nano, Shimadzu). RNA was then reverse-transcribed to complementary DNA using 2729 Thermal Cycler (Applied Biosystems). RT-qPCR was carried out by 7300 Real Time PCR System (Applied Biosystems) and SYBR Green (KAPA Biosystems) was used to detect PCR products. Each sample was run in duplicate. Target gene mRNA expression levels were normalised to endogenous ribosomal S29 expression and compared with the average ΔCt value of CRM WT samples serving as a calibrator. Data are presented as relative transcript levels ($2^{-\Delta\Delta Ct}$) to show the fold change in gene expression. Primer sequences for mouse genes are shown in Supplementary Table 1.

Ex vivo vascular studies

Rapidly after euthanasia, the thoracic aorta of male mice was placed in ice-cold oxygenated Krebs solution (0.119 mol l^{-1} NaCl, 0.025 mol l^{-1} $NaHCO_3$, 0.0055 mol l^{-1} glucose, 0.0047 mol l^{-1} KCl, 0.0012 mol l^{-1} KH_2PO_4, 0.0012 mol l^{-1} $MgSO_4 \cdot 7H_2O$ and 0.0025 mol l^{-1} $CaCl_2 \cdot 2H_2O$). Dissected 2 mm aortic ring segment was mounted in a wire-myograph system (Danish Myograph Technologies, Aarhus, Denmark) as previously described (Rinne*, et al.* 2015). During the experiments, aortic segments were kept in aerated Krebs solution (95% O_2 and 5% CO_2) and heated to 37°C.

Isolated rings of aortae were contracted three times with 0.062 mol l^{-1} KCl to determine the maximal contraction of the vessel. $Alpha_1$-adrenoceptor-mediated vasoconstrictor responses were determined by cumulative doses of phenylephrine. Aorta was precontracted with 0.001

mol l^{-1} prostaglandin F_{2alpha} to obtain 50-80% of the maximal reference contraction to KCl and endothelium-dependent vasodilatation response to acetylcholine was determined. Endothelium-independent relaxation was studied in a similar fashion using cumulative doses of sodium nitroprusside (SNP). The contribution of NO to endothelium-dependent vasodilatation was determined by incubating the aortic ring with N^{omega}-Nitro-L-arginine (L-NNA, 0.0001 mol l^{-1}) 30 minutes before contracting the aorta with phenylephrine, and subsequently relaxing it with acetylcholine. Chart5 and PowerLab were used for data recording and analysis (ADInstruments, Colorado Springs, CO, USA).

Statistical analyses

Statistical differences were calculated by 2-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests when three or more groups were compared. Unpaired two-tailed t test was used when only two groups were compared. All statistical analyses were performed using GraphPad Prism versions 6.0 and 7.02. *P* values of less than 0.05 were considered statistically significant. All data are presented as mean ± standard error of the mean (SEM).

Results

MSH-OE improves glucose tolerance without affecting body weight or composition in *Ldlr*$^{-/-}$ mice

The melanocortin system regulates several physiological functions, including energy homeostasis. Hence, we first aimed to investigate whether transgenic MSH-OE affects body weight or composition, cholesterol levels or glucose tolerance in *Ldlr*$^{-/-}$ mice. Body weight was monitored weekly during the 16-week diet-intervention. In male mice, MSH-OE had no effect on the body weight development during the 16-week diet-intervention in either of the diet groups (Fig. 1A). However, female MSH-OE mice tended ($P = 0.07$) to have lower body weight throughout the diet intervention (Fig. 1B). Quantitative NMR scanning revealed a significantly lower fat mass, but not lean mass, in MSH-OE female mice (Fig. 1D-F). No genotype differences in fat or lean mass were observed in male mice (Fig. 1C-E). In line with these results, epididymal or retroperitoneal white adipose tissue (WAT) weights were not different in male mice (Table 1). In female MSH-OE mice, gonadal fat mass tended to be decreased (Table 1, $P = 0.06$). It was of note that MSH-OE restrained Western diet-induced increase in relative liver weight in male mice (65.4 ± 2.5 vs 57.9 ± 2.0 mg/g body weight, $P < 0.05$). This was further supported by the measurement of liver triglyceride content, which tended to be lower in Western diet-fed male MSH-OE mice and was significantly reduced in female MSH-OE mice (Supplementary Figure 2). Serum cholesterol and leptin levels were markedly increased by Western diet, but the levels were comparable between the genotypes (Table 1).

Interestingly, we found that MSH-OE attenuated the increase in blood glucose at 20 min time point after the glucose injection in both male and female Western diet-fed MSH-OE mice

compared with WT mice, indicating an improvement in glucose tolerance ($P < 0.01$ and $P < 0.0001$ for genotype effect, respectively, Fig. 2).

Decreased plaque accumulation in MSH-OE mice

The plaque accumulation in the aortic arch was quantified by *en face* Sudan IV stainings (Fig. 3). Western diet significantly increased the plaque deposition in the aortic arch (Fig. 3C, $P < 0.0001$), but more importantly, MSH-OE mice showed a significant decrease in the intimal plaque accumulation on Western diet in both male and female mice (Fig. 3C-F, $P = 0.03$ and $P = 0.02$, respectively).

To further characterise the atherosclerotic plaques, the lipid and collagen depositions in the aortic root were determined from Oil Red O and Masson's Trichrome stained histological sections (Fig. 4A). Consistent with the *en face* stainings of the aortic arch, the total lesion area in the aortic root tended to be reduced in male MSH-OE mice on Western diet (Fig. 4B), but the difference did not reach statistical significance ($P = 0.15$). Because a thin fibrotic cap is associated with vulnerable plaque phenotype, we evaluated the proportion of fibrotic tissue in the plaques by staining sections of aortic root with Masson's Trichrome. These results showed no difference in the collagen deposition between the genotypes (Fig. 4C). In line with this finding, MSH-OE had no effect on the aortic mRNA expression of *Col1a2* and *Col3a1* that code for collagen types 1 and 3, respectively (data not shown). Given that the monocytes and macrophages play a crucial role in atherosclerotic lesion formation, we sought to characterise the macrophage deposition and polarisation in the plaques of the aortic root. The absolute macrophage count and macrophage density in the intima, as visualised by Mac3 antibody, were unaltered between the genotypes on Western diet (Fig. 4D). However, we found a significant decrease in iNOS-positive area in the intima of MSH-OE mice compared with WT

mice (Fig. 4E, $P = 0.01$), indicating a decrease in the proportion of proinflammatory M1 macrophage phenotype in the aortic root.

MSH-OE attenuates aortic inflammation

To quantify the local expression of cytokines that promote the development of atherosclerosis, we performed RT-qPCR from the samples of the thoracic aortae. We found that the relative expression levels of *Il6*, *Ccl2* and *Ccl5* were substantially increased by Western diet, and that the increase of these cytokines were significantly attenuated in MSH-OE mice compared with WT mice (Fig. 5B-D, $P = 0.04$, $P = 0.003$ and $P < 0.0001$, respectively), indicating that MSH-OE alleviates the diet-induced increase of these proinflammatory cytokines. We also determined anti-inflammatory M2 macrophage markers *Cd206* and *Tgfb*, but found no genotype differences in these markers (Fig. 5E-F).

MSH-OE restrains alpha$_1$-adrenoceptor-mediated vasoconstriction and enhances endothelium-dependent vasodilation

As the endothelial dysfunction shifts the vascular tone towards vasoconstriction, we evaluated the functional properties of the aorta of MSH-OE mice using *ex vivo* wire-myograph system. First, we evaluated the contractile-responses to potassium and found that the potassium-evoked vasoconstrictions were unchanged between the genotypes, demonstrating an uncompromised maximum contractile capacity in MSH-OE mice (Fig. 6A). Of note, the aortae of MSH-OE mice were less sensitive to the cumulative doses of phenylephrine compared with those of WT mice, when mice were fed regular diet, indicating that MSH-OE restrained the alpha$_1$-adrenoceptor-mediated contractile-responses (Fig. 6B, $P = 0.0015$). However, on Western diet, there was no difference between the genotypes (Fig. 6C).

Next, we investigated the endothelium-dependent relaxation responses to acetylcholine and found that MSH-OE significantly enhanced the vasorelaxation responses in mice on regular diet (Fig. 7A). We also examined relaxation responses to acetylcholine after inhibiting the tissue nitric oxide synthase (NOS) activity with L-NNA and observed that the blunting of the overall vasodilation was more pronounced in MSH-OE mice in comparison with WT mice on regular diet (Fig. 7C and Supplementary Figure 3), suggesting that MSH-OE augments the NO-dependent component of the vasodilation. However, on the Western diet, the vasorelaxation responses before and after NOS inhibition were comparable between the genotypes (Fig. 7B and D). Furthermore, MSH-OE had no effect on the endothelium-independent relaxation responses to the NO donor SNP (Fig. 7E-F).

Discussion

The present study demonstrates for the first time that melanocortin activation attenuates the progression of murine atherosclerosis. Specifically, we found that MSH-OE improves glucose tolerance, decreases plaque accumulation in the aortic arch and suppresses the expression of pro-inflammatory cytokines. Moreover, MSH-OE improves the function of the aorta by resisting the phenylephrine-induced contraction and by enhancing the endothelium-dependent vasorelaxation during early atherosclerosis.

The melanocortin system, and especially MC4R in the central nervous system, is a major regulator of the energy homeostasis. In the present study, we found that MSH-OE had no effect on overall body weight development during 16-week diet-intervention, but decreased the proportional fat accumulation in females. High visceral fat mass has a close association with metabolic syndrome, insulin resistance and endothelial dysfunction, linking it with an increased risk of cardiovascular disease (Kim*, et al.* 2015, Rittig*, et al.* 2010). However, the current study revealed that adiposity was only modestly reduced in female MSH-OE mice and it was also the only parameter that showed sex-specific effect. Furthermore, serum leptin level, which closely correlates with body weight and with fat mass in particular, was unchanged in male and female MSH-OE mice. These observations highlight that MSH-OE is capable of reducing atherosclerosis independent of body weight and fat mass.

Importantly, we show that transgenic MSH-OE improved glucose tolerance in both male and female mice. Several studies have demonstrated the crucial role of the melanocortin system in glucose homeostasis and the current study consolidates this role by illustrating beneficial effects on glucose tolerance in a mouse model of atherosclerosis. Impaired glucose tolerance is a risk factor for atherosclerosis (Di Bonito*, et al.* 2016), and in fact, most diabetic patients

die of atherosclerotic complications (Beckman, *et al.* 2002). Even small increases in blood glucose predispose to cardiovascular complications (Alexander, *et al.* 2003), highlighting the importance of glucose homeostasis regulation in the prevention and treatment of atherosclerosis. In this study, transgenic MSH-OE prevented the diet-induced impairment in glucose handling, which is in line with previous evidence showing increased glucose uptake and insulin-sensitivity upon MC4R activation and in melanocortin overexpression models (Chai, *et al.* 2009, Lee, *et al.* 2007, Obici, *et al.* 2001, Savontaus, *et al.* 2004). Although central melanocortin activation has been shown to acutely increase gluconeogenesis in the liver (Gutierrez-Juarez, *et al.* 2004), the beneficial effects of melanocortins on glucose homeostasis in the chronic setting are primarily driven by improved insulin action on peripheral glucose uptake. This effect occurs independent of body weight or fat mass and likely involves also direct peripheral actions of melanocortins on the skeletal muscle (Enriori, *et al.* 2016, Obici, *et al.* 2001). In the current study, MSH-OE mice showed unchanged basal glucose levels but significantly improved glucose clearance 20 min after glucose injection, supporting the notion of enhanced glucose uptake as a primary mechanism of action. We also found that MSH-OE restrained Western diet-induced increase in hepatic fat accumulation, which is congruent with a study by Lee et al., who found that the liver weight and hepatic fat accumulation was markedly reduced in MSH-OE mice compared to WT mice on high-fat diet (Lee, *et al.* 2007). The reduced hepatic fat accumulation might in part explain the improvements in glucose tolerance and plaque accumulation in MSH-OE mice as non-alcoholic fatty liver disease is closely correlated with cardiovascular disease in type 2 diabetic patients (Targher, *et al.* 2005).

The most important finding of this study was that MSH-OE significantly reduced atherosclerosis in both male and female mice. The reduced plaque size was not associated

366 with signs of increased plaque stability as no changes were noted in plaque collagen content.
367 In our previous study, where we treated the atherosclerotic mice for 4 weeks with the alpha-
368 MSH analogue melanotan 2, there were no difference in the plaque size between melanotan 2
369 and vehicle-treated groups (Rinne*, et al.* 2014). However, this difference most probably stems
370 from the differences in the animal models used, i.e. melanocortin administration *versus*
371 transgenic melanocortin overexpression. Although melanotan 2 is a very potent alpha-MSH
372 analogue, the duration of active treatment (4 weeks) might be insufficient to limit the plaque
373 accumulation and promote regression of existing plaques, when the mice had already
374 developed advanced atherosclerosis before the treatment initiation. In the present study, on
375 the other hand, the transgenic MSH-OE provided a life-long MSH exposure, and therefore,
376 might be more efficient in limiting or even preventing the plaque accumulation and
377 development of glucose intolerance at the early stage of atherosclerosis. Moreover, the
378 transgenic model provides consistent and stable activation of the melanocortin system,
379 whereas the administration of MSH peptides requires frequent i.p. injections and therefore the
380 level of MSH peptides might vary significantly throughout the day, which might blunt their
381 therapeutic effects.
382
383 Cholesterol is an important risk factor and driving force of atherosclerosis development.
384 However, mounting evidence demonstrates that cholesterol triggers inflammation, which, in
385 turn, promotes atherosclerosis. Given that MSH-OE did not change plasma cholesterol
386 concentration, the reduced plaque accumulation in MSH-OE mice is likely explained by the
387 reduced pro-inflammatory cytokine levels in the aorta. Firstly, the expression of the
388 atherogenic CCL2 was downregulated in MSH-OE mice. The atherogenic effects of CCL2
389 stem from its ability to recruit monocytes into the inflammatory site, an effect that is mediated
390 by its cognate receptor CCR2 and abolished in *Ccr2* deficient mice (Boring*, et al.* 1997,

391 Boring*, et al.* 1998). Furthermore, *Ccl2* deficiency or loss-of-function polymorphism
392 decreases atherosclerosis both in mice and humans (Wan & Murphy. 2013). Secondly, MSH-
393 OE mice displayed markedly reduced Ccl5 mRNA levels. CCL5 and its receptors CCR1 and
394 CCR5 guide leukocyte entry into atherosclerotic plaques and promote atherosclerosis
395 (Drechsler*, et al.* 2015). For instance, *Ccr5* deficient bone transplantation in mice decreased
396 the plaque burden and monocyte trafficking to the sites of inflammation (Braunersreuther*, et*
397 *al.* 2007, Potteaux*, et al.* 2006). Thirdly, *Il6* was also decreased in the aorta of MSH-OE
398 mouse. IL6 is secreted by macrophages in the atherosclerotic plaques and especially,
399 macrophages loaded with free cholesterol are a major source of IL6 (Sukovich*, et al.* 1998).
400 Supporting the gene expression data, immunohistochemical stainings revealed that the
401 expression of the M1 type macrophage marker iNOS was reduced in the aortic root plaques of
402 MSH-OE mice. M1 macrophages feed plaque inflammation and vulnerability by secreting
403 proinflammatory markers such as CCL2, CCL5 and IL6 (Moore*, et al.* 2013). Taken together,
404 MSH-OE remarkably suppressed diet-induced arterial inflammation, which is likely to
405 contribute to the anti-atherosclerotic effects of transgenic melanocortin activation.
406
407 Because endothelial dysfunction causes imbalance in the vascular tone and contributes to the
408 pathogenesis of atherosclerosis, we evaluated the aortic constriction and dilation responses of
409 MSH-OE mice. We found that MSH-OE resisted the phenylephrine-induced vasoconstriction
410 and enhanced the endothelium-dependent relaxation in an *ex vivo* aorta on regular chow diet.
411 The enhanced relaxation response to acetylcholine was abolished by inhibition of NOS,
412 referring to an augmentation of NO availability in the aorta of MSH-OE mice. These findings
413 are well in line with our previous study, where treatment with alpha-MSH analogues
414 improved endothelial dysfunction in aged and diet-induced obese mice by increasing NO
415 availability (Rinne*, et al.* 2013). The lack of these effects in Western diet group might stem

from the fact that the diet is causing major endothelial dysfunction overruling the beneficial effects of MSH-OE. The restoration of endothelial function, and hence NO availability, is of importance because disturbed NO signalling plays a major role in the initiation of not only atherosclerosis but also of other cardiovascular diseases (Qian & Fulton. 2013). Furthermore, endothelium-derived NO controls leukocyte adhesion and supresses cytokine secretion in the vasculature and might thereby contribute to the observed decrease in pro-inflammatory cytokines in the aorta of MSH-OE mice (Davignon & Ganz. 2004). The present and previous evidence consolidate that MSH-OE ameliorates endothelial dysfunction by promoting endothelium-dependent vasodilation and vascular NO availability.

The transgenic MSH-OE mouse model provides clear advantages over conventional pharmacological models, such as stable MSH levels without the need for frequent and stressful peptide injections. A limitation of this study is that it is unable distinguish the contributions of alpha- and gamma$_3$-MSH and their respective receptors that mediate the observed anti-inflammatory and vasoactive effects. However, given that both alpha- and gamma$_3$-MSH display these effects, most probably, they both contribute. The receptors for alpha- and gamma$_3$-MSH, namely MC1R and MC3R, are widely distributed in the periphery and in the central nervous system and hence, the therapeutic effects of MSH-OE are likely to be mediated via both peripheral and central mechanisms. On the other hand, MC4R, being an important central regulator of energy and glucose homeostasis, has likely contributed to the metabolic phenotype of MSH-OE mice (Huszar*, et al.* 1997, Vaisse*, et al.* 2000). Recently, it was shown that deficiency of either MC1R or MC3R disturbs the anti-inflammatory signalling (Holloway*, et al.* 2015, Rinne*, et al.* 2015). However, it seems that MC3R plays a more significant role in the acute inflammatory response, whereas MC1R contributes more to the delayed immune response (Holloway*, et al.* 2015). Because both alpha- and gamma$_3$-

MSH have shown advantageous effects in inflammatory, metabolic and cardiovascular regulation, from a drug development point of view, it might be more beneficial to develop dual-agonists that would have more diverse therapeutic effects.

In conclusion, our study shows for the first time that melanocortin system activation protects against atherosclerosis by limiting vascular inflammation and by improving glucose tolerance. In line with previous evidence, we also show that MSH-OE protects the arterial endothelium, the dysfunction of which is a critical factor and an early marker of atherosclerosis. These findings emphasise that targeting of the melanocortin system might bring along wide-ranging therapeutic benefits. Given that atherosclerosis still places a global burden to the society, the melanocortin system could serve as a promising drug development target for immune-mediated vascular and metabolic disorders such as atherosclerosis.

Declaration of interest

The authors declare no conflict of interest.

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