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Minocycline reduces plaque size in diet induced atherosclerosis via p27^{Kip1}

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ABSTRACT

Objective: Minocycline, a tetracycline derivate, mediates vasculoprotective effects independent of its antimicrobial properties. Thus, minocycline protects against diabetic nephropathy and reduces neointima formation following vascular injury through inhibition of apoptosis or migration, respectively. Whether minocycline has an effect on primary atherogenesis remains unknown.
Methods: Using morphological and immunohistochemical analyses we determined *de novo* atherogenesis in ApoE−/− mice receiving a high fat diet (HFD) with or without minocycline treatment. The effect of minocycline on proliferation, expression of p27^{Kip1} or PARP-1 (Poly [ADP-ribose] polymerase 1), or on PAR (poly ADP-ribosylation) modification in vascular smooth muscle cells (VSMC) was analyzed in *ex vivo* and *in vitro* (primary human and mouse VSMC).
Results and conclusion: Minocycline reduced plaque size and stenosis in ApoE−/− HFD mice. This was associated with a lower number and less proliferation of VSMC, reduced PAR (poly ADP-ribosylation) modification and increased p27^{Kip1} expression within the plaques. In agreement with the *ex vivo* data minocycline reduced proliferation, PARP-1 expression, PAR modification while inducing p27 expression in human and mouse VSMC *in vitro*. These effects were observed at a low minocycline concentration (10 μM), which had no effect on VSMC migration or apoptosis. Minocycline inhibited PARP-1 and induced p27^{Kip1} expression in VSMC as efficiently as the specific PARP-1 inhibitor PJ 34. Knock down of p27^{Kip1} abolished the antiproliferative effect of minocycline. These data establish a novel antiatherosclerotic mechanism of minocycline during *de novo* atherogenesis, which depends on p27^{Kip1} mediated inhibition of VSMC proliferation.

1. Introduction

Minocycline, a tetracycline derivate, mediates cytoprotective effects unrelated to its antimicrobial properties. In particular, neuroprotective properties of minocycline have been demonstrated in a number of animal models of neurodegeneration or brain injury [1,2]. These studies have led to clinical evaluations of minocycline in patients with neuronal disease, which showed that minocycline has promising neuroprotective properties in humans [3]. Considering the potential long-term use of minocycline in patients with neurodegenerative disorders minocycline's effect on primary atherogenesis is relevant, but remains to be explored.

Recent studies demonstrated that minocycline conveys cytoprotective effects in vascular cells. For example, minocycline protects against diabetic microvascular complication [4,5]. In regard to macrovascular disease tetracycline derivates like minocycline or doxycycline reduce neointima formation following an acute vascular injury of the rat carotid artery [6]. In these studies the reduced number of vascular smooth muscle cells (VSMC) has been attributed to an inhibition of MMP activity and cytokine induced VSMC migration [6–8]. However, *in vitro* data demonstrated that minocycline directly reduces VSMC number, suggesting that minocycline may modulate cellular proliferation, although the underlying mechanism remains unknown [9–11].

Proliferation is increased in primary human atherosclerotic lesions, in particular in early lesions [12]. In undiseased arteries

Abbreviations: GOT, glutamate-oxalacetate transaminase (AST); GPT, glutamate pyruvate transaminase (ALT); HASMC, human aortic smooth muscle cell; HFD, high fat diet; MMP, matrix metalloproteinase; PAR, poly ADP-ribosylation; PARP-1, poly [ADP-ribose] polymerase 1; VSMC, vascular smooth muscle cell.

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VSMCs proliferate at a low frequency and are arrested in the G_0/G_1 phase of the cell cycle. Endothelial dysfunction or arterial injury induce proliferation of VSMCs [13]. Cell proliferation is negatively regulated by cyclin-dependent kinase (CDK) inhibitors (CKI), which associate with and inhibit the activity of CDK/cyclin holoenzymes, leading to a G_1 arrest [14]. The CKI $p27^{Kip1}$, which is expressed in healthy and atherosclerotic arteries, has evolved as an important regulator of VSMC proliferation [15]. Mice lacking $p27^{Kip1}$ display enhanced arterial cell proliferation and larger atherosclerotic plaques in a murine model of diet induced atherogenesis [12], establishing not only a role of $p27^{Kip1}$, but of proliferation in general during primary atherogenesis. Cell cycle progression is also regulated by PARP-1 (Poly [ADP-ribose] polymerase 1) [16], an enzyme potently inhibited by minocycline [17] and hence providing a potential mechanistic link between minocycline and regulation of proliferation.

Considering that minocycline has vasculoprotective effects we addressed the question whether minocycline modulates PARP-1 activity and proliferation during atherogenesis. To this end we induced atherogenesis using a high fat diet (HFD) in atherosclerosis prone ApoE-deficient (ApoE−/−) mice, treating a subgroup of mice for the entire study period with minocycline. We show that minocycline reduces plaque size and inhibits proliferation of smooth muscle cells through a PARP-1 and $p27^{Kip1}$ dependent mechanism.

2. Materials and methods

See the materials and methods section in Supplementary data for details.

2.1. Mice

Animal experiments were carried out in accordance with the local Animal Care and Use Committee (Regierungspraesidium Karlsruhe, Germany). ApoE−/− mice (C57Bl/6 background) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

Mice (age 6–8 weeks) received either a Western type diet or a normal chow diet (controls). A subset of the Western type diet-fed mice received minocycline intraperitoneally (5 mg/kg body weight) once per day from the first day of the diet until 1 day before analysis. Control mice were injected with an equal volume of PBS intraperitoneally once per day. Mice were sacrificed and analyzed after 20 weeks of treatment (see the materials and methods section in Supplementary data).

2.2. Analysis of blood lipids, cytokines, and transaminases

Plasma levels of lipids were measured as previously described using the Advia 2400 Chemistry System Siemens (Eschborn, Germany) [18]. Plasma levels of TNFα and IL-6 were determined by ELISA following manufacturer instructions (eBioscience, Germany). Plasma levels of GOT (glutamate-oxalacetate transaminase, AST, U/l) and GPT (glutamate pyruvate transaminase, ALT, U/l) were measured using a two step enzymatic reaction on the Advia 2400 Chemistry System.

2.3. Histology and immunohistochemistry

Thoracic aortas were stained with Oil-red-O. MOVATs stain was performed on frozen sections of brachiocephalic arteries. Samples were analyzed by a blinded investigator using the Image Pro Plus software [18] (see the materials and methods section in Supplementary data).

2.4. Cell culture

All experiments performed with human umbilical vein endothelial cells (HUVECs) or human aortic smooth muscle cells (HASMCs) were done with passages 4–6. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator using endothelial or smooth muscle cell growth medium in the presence of growth factors and supplements. Cells were subcultured at confluence by trypsinization with 0.05% trypsin and 0.02% EDTA. Medium was changed every other day. For additional information regarding THP-1 cells and primary mouse aorta VSMC see the materials and methods section in Supplementary data.

2.5. Apoptosis and proliferation assays

Apoptosis was determined by TUNEL assay [4]. Proliferation was determined *in vitro* by BrdU labelling (see the materials and methods section in Supplementary data).

2.6. Immunoblotting

Immunoblotting was essentially done as previously described [4] (see the materials and methods section in Supplementary data).

2.7. Statistical analyses

All *in vitro* experiments were performed at least in triplicates. The data are summarized as the mean ± s.e.m. (standard error of the mean). Statistical analyses were performed using Student's *t*-test, ANOVA, or χ^2 test (as indicated in the figure legend or the text). StatistiXL software (http://www.statistixl.com) was used for statistical analyses. Statistical significance was accepted at the $P < 0.05$ level.

3. Results

3.1. Minocycline reduces atherosclerotic plaque size and vascular stenosis

En face analysis of Oil-red-O stained thoracic aortas showed that minocycline significantly reduced the plaque burden of the thoracic aorta in ApoE−/− HFD mice, in particular in the portion of the aortic arch (7.1% vs. 11.1%, $P = 0.039$, Fig. 1A and B). Likewise, we observed a significant reduction of plaque burden within the aortic root of minocycline treated ApoE−/− HFD mice (plaque size $43.3 \times 10^3\ \mu m^2$ vs. $80.1 \times 10^3\ \mu m^2$, $P < 0.001$, Fig. 1C). Histological analysis of cross-sections of the truncus brachiocephalicus, which exhibits a highly consistent rate of lesion progression and develops characteristics of advanced lesions [19], likewise showed smaller plaques in minocycline treated ApoE−/− HFD mice in comparison to ApoE−/− HFD ($50.1 \times 10^3\ \mu m^2$ vs. $159.2 \times 10^3\ \mu m^2$ $P = 0.02$, Fig. 1D and E). The total vessel cross section area was increased in ApoE−/− HFD with and without minocycline treatment, reflecting positive vascular remodelling. Vascular remodelling was somewhat less pronounced in minocycline treated ApoE−/− HFD mice, but this difference was not significant ($181.1 \times 10^3\ \mu m^2$ vs. $253.1 \times 10^3\ \mu m^2$, $P = 0.192$, Fig. 1F). Vascular stenosis was less severe in ApoE−/− HFD diet receiving minocycline (34% vs. 59% in ApoE−/− HFD control mice, $P = 0.038$, Fig. 1G).

Treatment with minocycline was well tolerated and did not impair the apparent health or survival of the mice. Analysis of serum samples showed no significant differences of total cholesterol (439.0 mg/dl vs. 538.1 mg/dl, $P = 0.16$, Supplementary Fig. S1B), triglyceride (43.0 mg/dl vs. 44.1 mg/dl, $P = 0.16$, Supplementary Fig. S1A) or HDL levels (17.5 mg/dl vs. 14 mg/dl,