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PARP1 is required for adhesion molecule expression in atherogenesis

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Aims Atherosclerosis is the leading cause of death in Western societies and a chronic inflammatory disease. However, the key mediators linking recruitment of inflammatory cells to atherogenesis remain poorly defined. Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme, which plays a role in acute inflammatory diseases.
Methods and results In order to test the role of PARP in atherogenesis, we applied chronic pharmacological PARP inhibition or genetic *PARP1* deletion in atherosclerosis-prone *apolipoprotein E-deficient* mice and measured plaque formation, adhesion molecules, and features of plaque vulnerability. After 12 weeks of high-cholesterol diet, plaque formation in male *apolipoprotein E-deficient* mice was decreased by chronic inhibition of enzymatic PARP activity or genetic deletion of *PARP1* by 46 or 51%, respectively ($P < 0.05$, $n \geq 9$). PARP inhibition or *PARP*1 deletion reduced PARP activity and diminished expression of inducible nitric oxide synthase, vascular cell adhesion molecule-1, and P- and E-selectin. Furthermore, chronic PARP inhibition reduced plaque macrophage (CD68) and T-cell infiltration (CD3), increased fibrous cap thickness, and decreased necrotic core size and cell death ($P < 0.05$, $n \geq 6$).
Conclusion Our data provide pharmacological and genetic evidence that endogenous *PARP1* is required for atherogenesis *in vivo* by increasing adhesion molecules with endothelial activation, enhancing inflammation, and inducing features of plaque vulnerability. Thus, inhibition of PARP1 may represent a promising therapeutic target in atherosclerosis.

1. Introduction

Atherosclerosis is the primary cause of myocardial infarction and stroke and thereby the leading cause of death in Western countries. It is a chronic inflammatory disease resulting from the interaction between modified lipoproteins, activated endothelial cells, inflammatory cells, and elements of the arterial wall.[1] However, the key mediators of atherosclerosis that may represent effective therapeutic targets remain poorly defined.

Mammalian poly(ADP-ribose)polymerase 1 (PARP1) is an abundant nuclear chromatin-associated protein. It belongs to a large family of enzymes that catalyse the transfer of ADP-ribose units from their substrate β-nicotinamide adenine dinucleotide (NAD^+) covalently to themselves and other nuclear chromatin-associated proteins.[2,3] Although PARP1 protein synthesis can be regulated in response to several stimuli, PARP1 is principally regulated via substrate interactions through its catalytic site. PARP1 is activated by DNA strand breaks, and thus involved in DNA damage response pathways. In addition, PARP1 may induce cell dysfunction or necrosis by depleting cellular energy pools.[3]

In vivo experiments using *PARP1* deletion[4] or pharmacological inhibition of PARP demonstrate a role for PARP in *acute* inflammatory processes such as myocardial infarction,[5,6] diabetes-induced endothelial dysfunction in mice,[7] and lipopolysaccharide-induced septic shock.[8] In contrast, much less is known about its role in *chronic* inflammatory diseases.

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As inflammation is crucially involved in atherosclerosis,[1] we hypothesized that *PARP1* is required for the formation of atherosclerotic plaques. Thus, we examined the effects of chronic PARP inhibition (by treatment with PJ34) and genetic *PARP1* deletion on plaque formation in atherosclerosis-prone *apolipoprotein E* knockout (*ApoE*$^{-/-}$) mice.[9] We found that chronic pharmacological PARP inhibition and genetic deletion of *PARP1* decreased atherosclerosis, reduced enzymatic PARP activity, and diminished expression of inducible nitric oxide synthase (iNOS) and adhesion molecules. Furthermore, pharmacological PARP inhibition reduced the number of plaque inflammatory cells and diminished features of plaque vulnerability.

2. Methods

2.1 Animals

Experiments with the PARP inhibitor PJ34 were performed using *ApoE*$^{-/-}$ mice[9] on a pure C57Bl/6J background. For the genetic experiments, *PARP1*$^{-/-}$[4] mice (129SvEv background) were crossbred with *ApoE*$^{-/-}$ mice (C57Bl/6J background) in order to obtain *ApoE*$^{-/-}$*PARP1*$^{+/+}$ and *ApoE*$^{-/-}$*PARP1*$^{-/-}$ mice that all had the identical mixed 129SvEv/C57Bl/6J background.

2.2 Mouse experiments and poly(ADP-ribose) polymerase inhibitor

Male *ApoE*$^{-/-}$ mice[9] or *ApoE*$^{-/-}$*PARP1*$^{+/+}$ ($n = 9$) and *ApoE*$^{-/-}$*PARP1*$^{-/-}$ ($n = 10$) mice were fed a high-cholesterol diet (CD; 1.25% total cholesterol, D12108, Research Diets, New Brunswick, NJ, USA) for 3 months starting at the age of 8 weeks. The pharmacological experiments were carried out as follows: After 4 weeks of diet, PJ34 [N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,Ndimethylacetamide, EMD Biosciences, San Diego, CA, USA], was administered via subcutaneous implantation of a custom-made pellet (Innovative Research of America, Sarasota, FL, USA); this pellet provided a continuous release of 250 μg PJ34/day for 60 days ($n = 25$). We selected this dosage based on previous studies that provided PJ34 parenterally up to 3 weeks.[10] Mice with implantation of pellets containing vehicle (placebo, $n = 25$) as well as age-matched C57Bl/6J wild-type mice ($n = 26$) served as negative controls. At 20 weeks of age, i.e. 8 weeks after pellet implantation, all animals were euthanized with an overdose of isoflurane (Abbott; Baar, Switzerland) inhalation for tissue harvesting. For the genetic experiments, wild-type mice with a 129SvEv/C57Bl/6J background were used as a control group. All animal experiments were approved by the local animal committee and performed in accordance with our institutional guidelines that conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.3 Blood chemistry and haematology

At the time of tissue harvesting, 0.5 to 1 ml of blood was drawn from the right ventricle with heparinized syringes, immediately centrifuged at 4°C, and the plasma stored at −80°C. Total cholesterol, triglycerides, and free fatty acids were analysed using the reagents TR13421, TR22421 (both Thermo Electron Clinical Chemistry & Automation Systems) and 994–75409 (Wako Chemicals GmbH, Neuss, Germany), as recommended by the manufacturer. The lipid distribution in plasma lipoprotein fractions was assessed by fast-performance liquid chromatography gel filtration with a Superose 6 HR 10/30 column (Pharmacia, Uppsala, Sweden).[11] Plasma values of vascular cell adhesion molecule 1 (VCAM-1) (MVC00) and intracellular adhesion molecule 1 (ICAM-1) (MIC100; both R&D Systems, Inc., Minneapolis, MN, USA) were determined using ELISA, and monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin 6 (IL-6) using multiplex array systems (BD FACSArray Bioanalyzer System and Becton Dickinson Cytometric Bead Array Mouse Inflammation Kit 552364; BD Biosciences, Erembodegem, Belgium). For haematologic analyses, blood drawn from the right ventricle was transferred to vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed within 6 h.

2.4 Tissue harvesting and processing

After puncturing the left ventricle and cutting the right atrium, vessels were rinsed briefly with normal saline and the aorta was excised after removing adventitial tissue and fat. For *en face* analyses, thoraco-abdominal aortae (from the left subclavian artery to the iliac bifurcation) were opened longitudinally.[12] For histological examination, the aortic arch was embedded in OCT (optimal cutting temperature) compound (Tissue-Tek; Sakura, The Netherlands), frozen on dry ice, and stored at −80°C. For biochemical analyses, aortic arches were shock-frozen in liquid nitrogen and stored at −80°C.

2.5 Western blotting

This technique was performed according to standard procedures including stripping and re-probing of membranes as needed. The following primary antibodies were used: polyclonal rabbit antibodies against PARP1 (ALX-210–619; Alexis Corporation, Lausen, Switzerland) and monoclonal mouse antibodies against poly-ADP-ribose (ALX-210–890; Alexis) as well as iNOS (clone 6; BD Biosciences Franklin Lakes, NJ, USA); primary monoclonal rat antibodies were applied to detect CD106 (VCAM-1, clone MCA1229; Serotec, Oxford, UK), CD62E (E-selectin, clone 10E9.6), CD62P (P-selectin, clone RB40.34; both BD Biosciences Pharmingen), and α-tubulin (clone B512; Sigma Chemical Co., St Louis, MO, USA). Secondary peroxidase-labelled anti-species-specific IgG antibodies (Amersham Biosciences, GE Healthcare Europe GmbH, Otelfingen, Switzerland) were diluted 1 : 4000 and incubated with the membranes for 1 h at room temperature (RT). Proteins were visualized after incubation of the membranes in SuperSignal West Femto Maximum Sensitivity Substrate for 5 min at RT. For PARP1 and α-tubulin, *enhanced luminescent substrate* was used (Pierce Biotechnology, Inc., Lausanne, Switzerland). Protein expression was quantified using Scion Image™ and expressed as ratio to tubulin or PARP1, respectively, after normalizing to WT values.

2.6 *En face* plaque staining

For quantification of atherosclerotic plaques, *en face* analysis of the thoraco-abdominal aorta was performed as described.[12] Plaque area was visualized by fat staining (Oil-red O), photographed with a digital camera (Olympus DP70, 12.5 megapixels) that was mounted on an Olympus SZX951 microscope (Olympus Schweiz AG, Schwerzenbach, Switzerland), and quantified (Analysis 5; SoftImaging System, Munster, Germany).[13]

2.7 Histological examination

Plaque size and composition were analysed in at least four serial longitudinal cryosections of the aortic arch (5 μm thickness, 50 μm intervals) that were thaw-mounted on glass slides (SuperFrostPlus; Menzel GmbH und Co., Braunschweig, Germany). A segment of the lesser curvature of the aortic arch was identified by dropping a perpendicular axis from the beginning of the innominate artery to the lesser curvature and following a 3-mm distance distal from this axis as described (see Supplementary material online, *Figure S1*).[14] Fat content was measured by ORO staining. Collagen, fibrous cap thickness, and necrotic core size were analysed by Elastica van Gieson (EVG) staining as described.[15] All sections were photomicrographed with a digital camera (Olympus

DP50-CU) mounted on an Olympus BX51 microscope, digitized and analysed (Analysis 5, SoftImaging System).

2.8 Immunohistochemistry and immunofluorescence

Acetone-fixed and rehydrated cryosections were incubated with primary antibodies for 30 min at RT. The staining of CD106, CD62E, and CD62P were performed with the same primary antibodies as used for western blotting. Primary rat monoclonal antibodies were used to stain the following antigens: CD68 (FA-11; Serotec), CD3 (KT3; Chemicon/Millipore, Billerica, MA, USA) and proliferation cell nuclear antigen (PCNA) (PC10, Abcam, Cambridge, UK). iNOS was detected with polyclonal rabbit antibodies (Serotec). Primary antibodies were revealed by goat anti-species-specific immunoglobulin antibodies, followed by alkaline phosphatase-labelled donkey antibodies against goat immunoglobulins. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. Colour reactions were performed at RT for 15 min with reagents from Sigma. Sections were counterstained with haemalum and coverslips mounted with glycerol and gelatine. Proliferation was determined by indirect fluorescent staining for PCNA using a mouse monoclonal antibody (clone PC10; DakoCytomation, Glostrup, Denmark) followed by an Alexa Fluor 488 conjugated anti-mouse antibody as secondary reagent. Cell death was assessed via terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL; Roche, Rotkreuz, Switzerland).[16] Nuclei were stained by 4′-6-diamidino-2-phenylindole (DAPI, Boehringer, Mannheim).

2.9 Statistics

Values are expressed as mean ± SEM. An unpaired Student *t*-test or ANOVA with post hoc Tukey's test was used to compare results between different groups. Significance was accepted at the level of $P < 0.05$.

3. Results

3.1 Plaque formation is reduced by chronic poly(ADP-ribose) polymerase inhibition or poly(ADP-ribose) polymerase 1 deletion

We examined the effects of chronic pharmacological inhibition of PARP (by treatment with the PARP inhibitor PJ34) and genetic *PARP1* deletion on plaque formation in atherosclerosis-prone *apolipoprotein E* knockout ($ApoE^{-/-}$) mice.[9] After 12 weeks of a high-cholesterol diet, the average *en face* plaque area in $ApoE^{-/-}$ mice treated with PJ34 for 8 weeks was reduced by 46% compared with the plaque area in placebo-treated $ApoE^{-/-}$ mice ($n = 10$, $P = 0.002$, *Figure 1A* and *B*). Similarly, atherosclerosis in $PARP1^{-/-}ApoE^{-/-}$ mice decreased by 51% ($n = 10$, $P = 0.039$) compared with $PARP1^{+/+}ApoE^{-/-}$ mice ($n = 9$; *Figure 1C* and *D*). $ApoE^{-/-}$ mice treated with PJ34 appeared healthy with the exception of 5 out of 25 mice those developed skin lesions; $ApoE^{-/-}$ mice with or without *PARP1* deletion were healthy with the exception of 1 out of 10

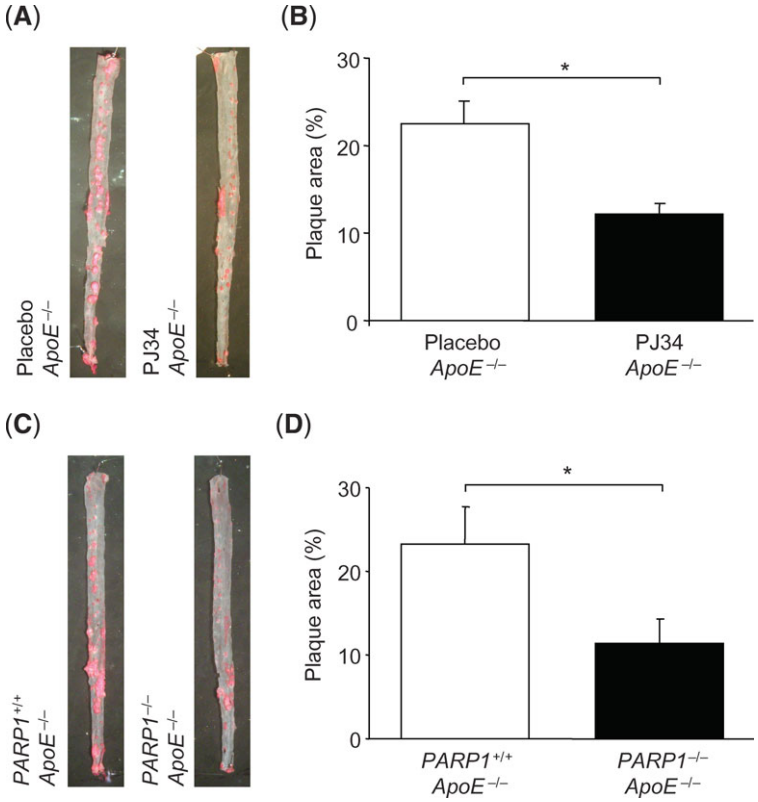

Figure 1 Aortic plaque formation is reduced by chronic poly(ADP-ribose) polymerase (PARP) inhibition or *PARP1* deletion in *apolipoprotein E* knockout ($ApoE^{-/-}$) mice. (*A*) *En face* fat staining of thoraco-abdominal aortae from $ApoE^{-/-}$ mice after 12 weeks of high-cholesterol diet; 8 weeks prior to harvesting, mice received placebo or PJ34. (*B*) Plaque formation was significantly decreased in PJ34-treated $ApoE^{-/-}$ mice ($n = 10$) compared with placebo-treated $ApoE^{-/-}$ mice ($n = 10$, $^*P = 0.002$). (*C*) Fat staining of thoraco-abdominal aortae from $PARP1^{+/+}ApoE^{-/-}$ or $PARP1^{-/-}ApoE^{-/-}$ mice after 12 weeks of high-cholesterol diet. (*D*) Plaque formation was significantly reduced in $PARP1^{-/-}ApoE^{-/-}$ mice ($n = 10$) compared with $PARP1^{+/+}ApoE^{-/-}$ mice ($n = 9$, $^*P = 0.039$); unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM.

PARP1$^{-/-}$*ApoE*$^{-/-}$ mice that developed a skin lesion (see Supplementary material online, *Figure S5*).

3.2 Poly(ADP-ribose) polymerase 1 activity in atherosclerotic aortae is diminished by chronic poly(ADP-ribose) polymerase inhibition or poly(ADP-ribose) polymerase 1 deletion

To ensure that effective PARP I inhibition or *PARP1* deletion was achieved in the target tissue, we analysed lysates of aortic arches from a subset of the above mice for PARP1 expression and PARP activity. PARP1 expression did not differ between wild-type mice and placebo- or PJ34-treated *ApoE*$^{-/-}$ mice (*Figure 2A*). However, PARP1 activity was increased by a factor of three in aortae from placebo-treated *ApoE*$^{-/-}$ mice compared with wild-type mice ($P < 0.001$), and markedly reduced in mice upon chronic treatment with PJ34 ($P < 0.05$ vs. wild-type and $P < 0.001$ vs. placebo) (*Figure 2A* and *B*). Similarly, aortic lysates from *PARP1*$^{+/+}$*ApoE*$^{-/-}$ mice exhibited increased PARP activity compared with lysates from wild-type mice. PARP activity and PARP1 expression were not detectable in *PARP1*$^{-/-}$ *ApoE*$^{-/-}$ mice. (*Figure 2C* and *D*).

3.3 Plasma lipid distribution upon chronic poly(ADP-ribose) polymerase inhibition or poly(ADP-ribose) polymerase 1 deletion

In order to investigate PARP-mediated effects on plasma cholesterol, we measured levels of total cholesterol and subfractions. *ApoE*$^{-/-}$ mice on a high-cholesterol diet displayed markedly increased total cholesterol levels compared with wild-type mice (see Supplementary material online, *Table S1*and *Figure S2A*). However, there was no significant difference between PJ34- and placebo-treated *ApoE*$^{-/-}$ mice. Interestingly, total cholesterol levels were increased in *PARP1*$^{-/-}$*ApoE*$^{-/-}$ compared with *PARP1*$^{+/+}$ *ApoE*$^{-/-}$ mice. This increase affected particularly the intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) subfractions (see Supplementary material online, *Table S2* and *Figure S3A*).

3.4 Expression of adhesion molecules in atherosclerotic aortae is decreased by chronic poly(ADP-ribose) polymerase inhibition or poly(ADP-ribose) polymerase 1 deletion

To investigate the putative mechanisms mediating reduced plaque formation in PJ34-treated or *PARP1*-deficient atherosclerotic mice, we analysed protein expression of adhesion molecules in aortic arch lysates from the same groups of animals. E-selectin, P-selectin, VCAM-1, and iNOS were barely detectable in wild-type mice without atherosclerosis, but their expression was significantly induced in placebo-treated *ApoE*$^{-/-}$ mice with abundant atherosclerotic plaques (*Figure 3A* and *B*). Chronic treatment with PJ34 markedly decreased E-selectin, P-selectin, VCAM-1, and iNOS protein levels (*Figure 3A* and *B*). Complementary immunohistochemical stainings of aortic arches confirmed these findings (see Supplementary material online, *Figure*

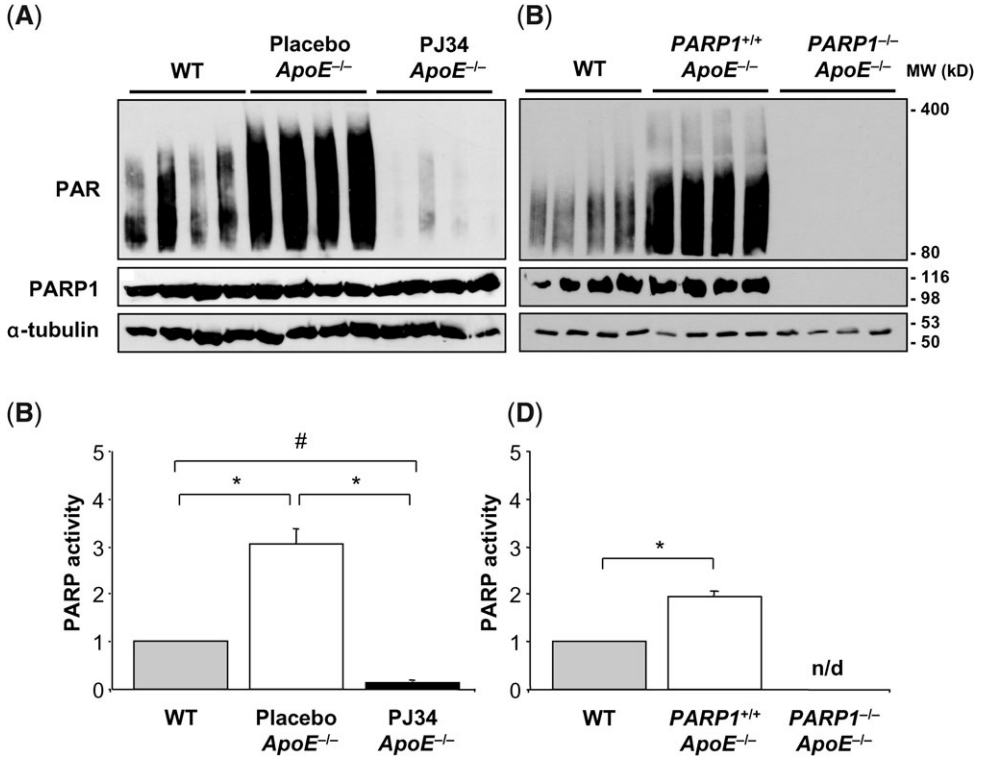

Figure 2 Poly(ADP-ribose) polymerase (PARP) activity in atherosclerotic aortae is diminished by chronic PARP inhibition or *PARP1* deletion. (*A* and *B*) Western blots and graph showing PAR [poly(ADP)-ribose; a measure of PARP activity] and PARP1 protein in aortic arch lysates obtained from wild-type (WT), placebo-, or PJ34-treated *ApoE*$^{-/-}$ mice; α-tubulin served as internal control for protein loading. PARP activity is given as a ratio to PARP1 protein and normalized to WT values. $n = 4$ in each group. *$P < 0.001$; #$P < 0.05$. (*C* and *D*) Western blots and graph showing PAR and PARP1 protein in aortic arch lysates obtained from WT, *PARP1*$^{+/+}$*ApoE*$^{-/-}$, or *PARP1*$^{-/-}$*ApoE*$^{-/-}$ mice; α-tubulin served as internal control for protein loading. PARP activity is given as a ratio to PARP1 protein and normalized to WT values. $n = 4$ in each group. *$P < 0.001$; n/d, not detectable. One-way ANOVA with post hoc Tukey's test. Values are expressed as mean ± SEM.

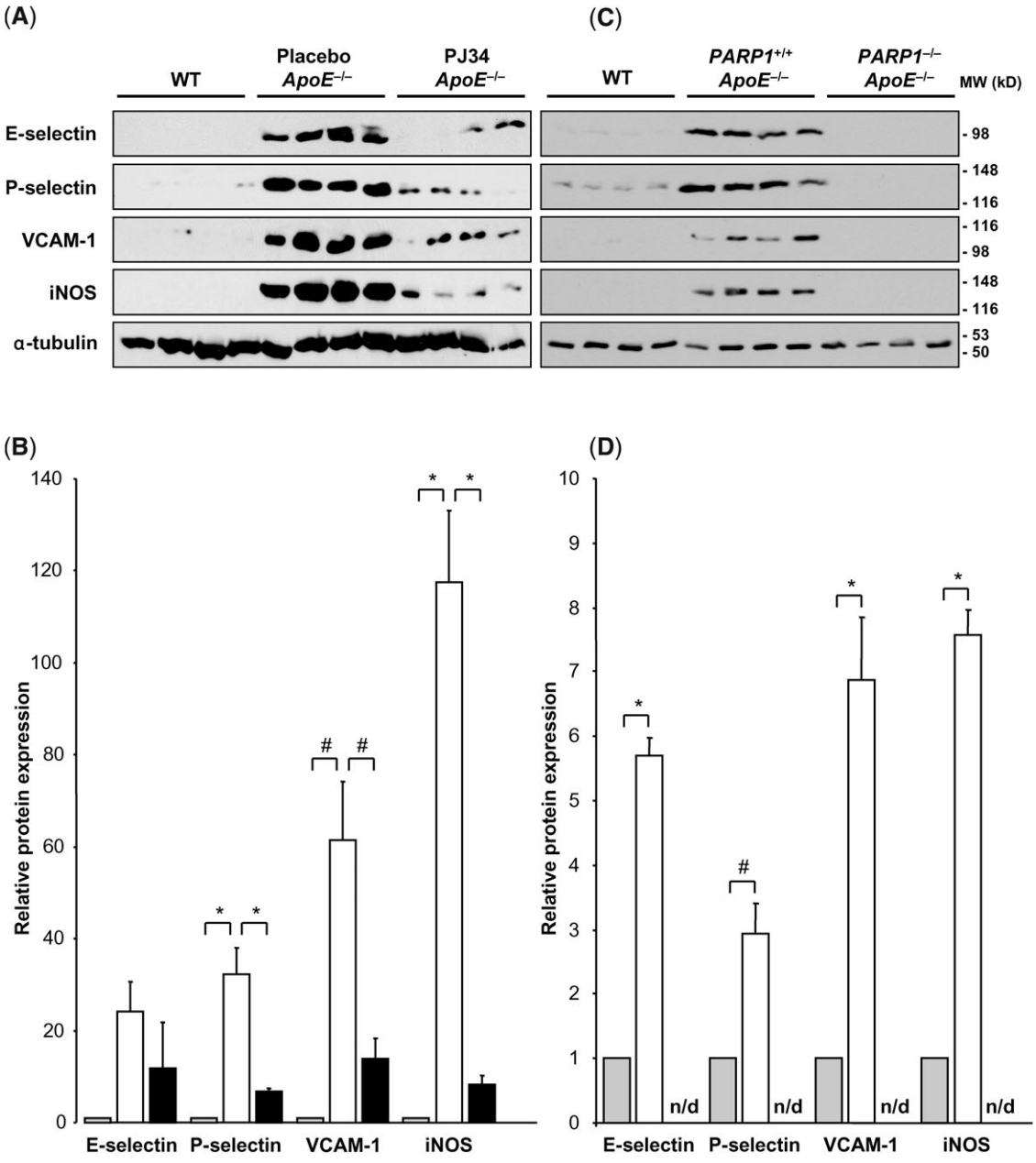

Figure 3 Expression of adhesion molecules in atherosclerotic aortae is decreased by chronic poly(ADP-ribose) polymerase (PARP) inhibition or *PARP1* deletion. (*A*) Western blots of E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1), and nitric oxide synthase (iNOS) in aortic arch lysates obtained from wild-type (WT), placebo-, or PJ34-treated *apolipoprotein E* knockout ($ApoE^{-/-}$) mice; $n = 4$ in each group. (*B*) Quantification of protein expression is given as ratio to α-tubulin (loading control) and normalized to WT. (*C*) Western blots of E-selectin, P-selectin, VCAM-1, and iNOS in aortic arch lysates obtained from WT, $PARP1^{+/+}ApoE^{-/-}$, or $PARP1^{-/-}ApoE^{-/-}$ mice. (*D*) Quantification of protein expression is given as ratio to α-tubulin (loading control) and normalized to WT; $n = 4$ in each group. *$P < 0.001$; #$P < 0.01$; n/d, not detectable. One-way ANOVA with post hoc Tukey's test. Values are expressed as mean ± SEM.

S4). In parallel, increased expression of E-selectin, P-selectin, VCAM-1, and iNOS was observed in aortic arch lysates from $PARP1^{+/+}ApoE^{-/-}$ mice, whereas these proteins were not detectable in $PARP1^{-/-}ApoE^{-/-}$ mice (*Figure 3C* and *D*).

3.5 Number of inflammatory cells and features of plaque vulnerability are reduced by chronic poly(ADP-ribose) polymerase inhibition

Inflammation is not only crucial in the early stages of atherogenesis related to plaque initiation, but also contributes to progression of late atherosclerotic events implicated in advanced lesions and plaque vulnerability.[17] Therefore, we quantified plaque inflammation in longitudinal sections of aortic arches of placebo- ($n = 9$) or PJ34-treated ($n = 6$) $ApoE^{-/-}$ mice (see Supplementary material online, *Figure S1*). Compared with placebo administration, chronic PJ34 treatment significantly decreased plaque areas (Oil-red O-stained areas, $P = 0.021$; *Figure 4A*), plaque macrophages (CD68-positive areas, $P = 0.018$; *Figure 4B*, and T-cells (CD3-positive areas, $P = 0.043$; *Figure 4C*).

To address whether PARP affects plaque stability, we assessed parameters of plaque vulnerability in serial

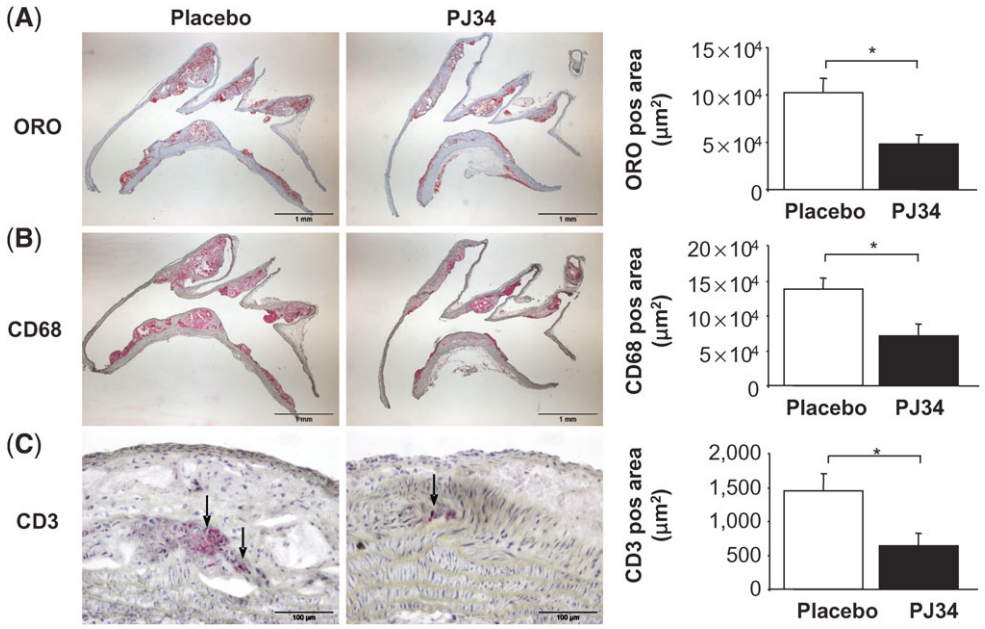

Figure 4 Number of inflammatory cells is reduced by chronic poly(ADP-ribose) polymerase (PARP) inhibition. Longitudinal aortic arch sections of placebo- ($n = 9$) or PJ34-treated ($n = 6$) *apolipoprotein E* knockout (*ApoE*$^{-/-}$) mice (left panels) stained with (*A*) oil-red O (ORO), (*B*) antibodies against CD68, or (*C*) antibodies against CD3 (arrows pointing to positive stainings), with the corresponding quantifications (right panels). *$P < 0.05$; unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM.

longitudinal sections of aortic arches in placebo- or PJ34-treated *ApoE*$^{-/-}$ mice. Compared with placebo administration, chronic PJ34 treatment increased fibrous cap thickness ($P = 0.023$) and reduced the necrotic core diameter ($P = 0.028$; *Figure 5A*). Furthermore, there was a trend to increased collagen content of atherosclerotic plaques in PJ34-treated compared with placebo-treated *ApoE*$^{-/-}$ mice ($P = 0.051$; *Figure 5A*). Cell death was lower in PJ34-treated *ApoE*$^{-/-}$ mice ($P = 0.027$; *Figure 5B*), whereas proliferation remained unchanged (data not shown).

3.6 Systemic mediators of inflammation are not affected by chronic poly(ADP-ribose) polymerase inhibition

To compare above local with systemic parameters of inflammation, we determined levels of plasma inflammatory cytokines and blood haematology counts. Plasma analyses of the inflammatory markers MCP-1, IFN-γ, IL-6, VCAM-1, ICAM-1, and TNF-α showed no significant differences between placebo- and PJ34-treated *ApoE*$^{-/-}$ mice, or between *PARP1*$^{+/+}$*ApoE*$^{-/-}$ and *PARP1*$^{-/-}$*ApoE*$^{-/-}$ mice. Overall blood leukocyte counts as well as blood monocytes, lymphocytes, and neutrophils were unchanged (see Supplementary material online, *Tables S3* and *S4*).

4. Discussion

Our results provide both pharmacological and genetic evidence that PARP1 increases expression of adhesion molecules, promotes plaque inflammation, and induces features of plaque vulnerability.

Specifically, we show that pharmacological inhibition of PARP via chronic PJ34 administration as well as genetic deficiency of *PARP1* markedly decreased plaque formation in atherosclerosis, a *chronic* inflammatory disease. Our findings match previous reports that demonstrate the importance of PARP1 for *acute* inflammatory diseases.[5,7,8,18,19] A most recent report shows beneficial effects of pharmacological PARP inhibition and *PARP1* deletion on the development of atherosclerotic plaques in mice.[20] The authors describe an inhibition of MCP-1 expression in atherosclerotic murine aortae, via an NFκB-dependent pathway. Beyond this study, we identified adhesion molecules as critical mediators of PARP inhibition or *PARP1* deletion, leading to a reduction in inflammatory activity within the atherosclerotic plaque.

Our results demonstrate that PARP activity was enhanced in aortae of atherosclerotic mice compared with non-diseased arteries of wild-type mice. Overall PARP activity in atherosclerotic aortae was markedly decreased upon chronic unspecific PARP inhibition with PJ34 and was not detectable in *PARP1*$^{-/-}$*ApoE*$^{-/-}$ mice. Our observations further show a specific increase in PARP1 activity in atherogenesis. They suggest a subordinate role for other PARP isoforms in this process given the lack of PARP activity (generated by PARP isoforms other than PARP1) in *PARP1*$^{-/-}$*ApoE*$^{-/-}$ mice. Thus, PARP1 is likely to be responsible for most of the effects attributed to the PARP family in atherogenesis. PARP is known to be activated via DNA damage mediated by free radicals and reactive oxygen species. Reactive oxygen species are widely present in inflammatory conditions such as atherosclerosis, and are therefore likely to be one of the key triggers of PARP activation in atherosclerotic lesions.

Interestingly, PARP1 expression remained similar in non-diseased arteries from wild-type mice and in aortae from atherosclerotic mice treated with the PARP inhibitor or placebo. As expected, PARP1 expression was undetectable in *PARP1*$^{-/-}$*ApoE*$^{-/-}$ mice. Enhanced PARP1 expression

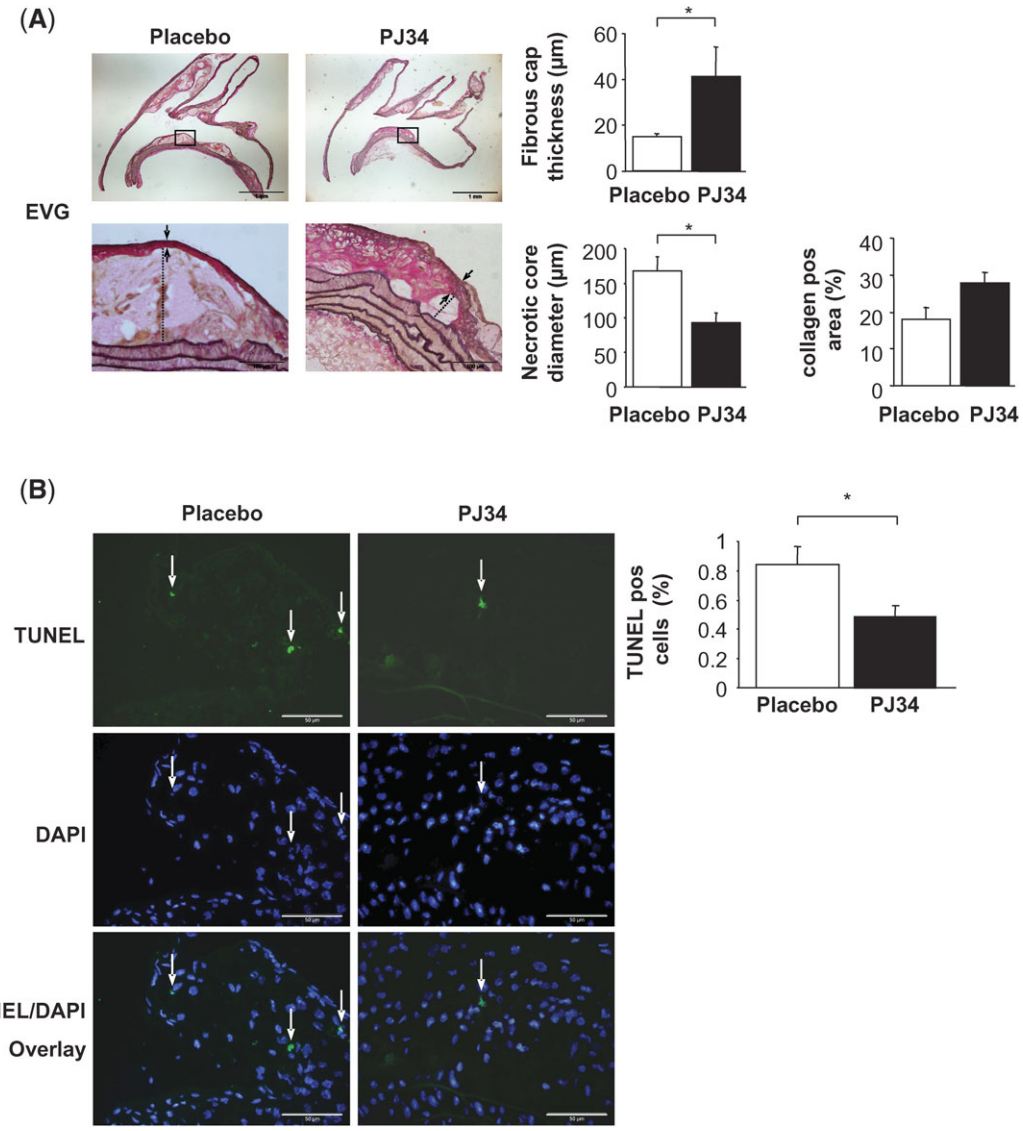

Figure 5 Features of plaque vulnerability are diminished by chronic poly(ADP-ribose) polymerase (PARP) inhibition. Aortic arch sections of placebo- ($n = 12$) or PJ34-treated ($n = 12$) *ApoE*$^{-/-}$ mice stained with (*A*) Elastica van Gieson (EVG); quantifications of fibrous cap thickness (arrows), necrotic core diameter, *$P <$ 0.05 (dashed line, bottom), and collagen content ($P = 0.051$). Aortic arch sections of placebo- or PJ34-treated *ApoE*$^{-/-}$ mice stained with (*B*) TUNEL, $P = 0.027$ [arrows indicating TUNEL-positive nuclei and their complementary 4′-6-diamidino-2-phenylindole (DAPI) stainings in the same sections] and the corresponding quantifications; unpaired two-tailed Student *t*-test. Bars = 50 µm. Values are expressed as mean ± SEM.

has been described in human atherosclerotic specimens,[21] but this discrepancy may be explained by species differences.

High levels of plasma triglycerides or cholesterol, in particular LDL cholesterol, are established clinical risk factors and key modulators of atherosclerosis.[22] Interestingly, total cholesterol levels were increased in *PARP1*$^{-/-}$ *ApoE*$^{-/-}$ compared with *PARP1*$^{+/+}$*ApoE*$^{-/-}$ mice. We consider this effect not relevant for atherogenesis as *PARP1*$^{-/-}$ *ApoE*$^{-/-}$ mice exhibited less atherosclerosis. Furthermore, pharmacological inhibition of PARP activity did not affect the lipid profile. It is known that a therapeutic agent may reduce atherogenesis independent of serum lipid levels. For example, Steffens *et al.*[23] have found a reduction in the progression of atherosclerosis in *ApoE*$^{-/-}$ mice treated with low dose of the phytochemical tetrahydrocannabinol. As in our study, this decrease in atherosclerosis was independent of serum cholesterol and triglyceride levels.

PARP inhibition decreases the expression of pro-inflammatory genes such as inducible iNOS and VCAM-1 in acute inflammatory conditions.[10] Therefore, we studied inflammatory mediators such iNOS, VCAM-1, P-, and E-selectin in atherosclerotic *ApoE*$^{-/-}$ mice to delineate possible mechanisms of PARP-dependent atherogenesis. The expression of these mediators was markedly decreased by chronic pharmacological PARP inhibition and virtually abolished in the presence of *PARP1* deficiency. Our results suggest that inhibition of PARP or deletion of *PARP1* reduce plaque initiation and progression in atherosclerosis-prone mice by decreasing expression of adhesion molecules and iNOS. This setting would inhibit the recruitment of monocyte-derived macrophages and T-cells to atherosclerotic plaques. The importance of P-selectin,[24] E-selectin,[25] VCAM-1,[26] and iNOS[27] in atherogenesis is well documented. We propose that activation of these adhesion molecules may depend, at least in part, on PARP as a co-activator in

NF-κB-mediated transcription.[28,29] Because NF-κB is induced in atherosclerosis,[1] our findings suggest that PARP1 may increase induction of adhesion molecules in atherosclerotic plaques via NF-κB.

In accordance with the decreased expression of adhesion molecules by either chronic PARP inhibition or *PARP1* deletion, we found a marked reduction of macrophages and T-cells in atherosclerotic plaques. These findings suggest that chronic pharmacological PARP inhibition reduces inflammatory cell recruitment resulting in fewer inflammatory cells in the subintimal space of aortae in *ApoE*$^{-/-}$ mice. In agreement, PARP inhibition has been shown to decrease the infiltration of neutrophils in response to acute zymosan-induced inflammation in mice and rats.[30] Our blood counts and plasma cytokines imply that the changes in adhesion molecules and inflammatory cells in atherosclerotic aortae did not affect systemic parameters of inflammation. We speculate that the critical changes in PARP activity occur in endothelial cells and monocytes/macrophages, thereby leading to the decrease in adhesion molecules and iNOS in atherosclerotic lesions. The reduction of PARP activity may also affect progression of atherosclerosis via secondary mechanisms, independent of adhesion molecule expression.

The reduced inflammation in response to chronic PARP inhibition is likely to explain the increased fibrous cap thickness and smaller necrotic core that are both features of increased plaque stability. In accordance with these findings, genetic deletion of the immune mediator CD156 or its inhibition by anti-CD40 antibody decreases atherosclerosis progression and augments atherosclerotic plaque stability by decreasing plaque leukocyte content.[14,15] In addition, we found reduced TUNEL-positive cells in advanced plaques of atherosclerotic mice treated with the PARP inhibitor. These findings suggest that increased PARP1 activity facilitates cell death in murine atherosclerotic lesions. In fact, in a stroke model, *PARP1*-deficient mice exhibit a marked reduction in cortical necrosis.[31] Complementary to our findings, increased smooth muscle cell death in murine atherosclerotic plaques induces features of plaque vulnerability.[32] These findings imply that chronic PARP inhibition enhances features of plaque stability.

While most groups applying PJ34 or other PARP inhibitors have given the drug parenterally, oral application of PJ34 has also been demonstrated to protect against the deleterious effects of various inflammatory diseases.[8,10] Therefore, we conclude that PJ34 or related compounds are absorbable if given orally and we extrapolate that they should be effective when given to humans. However, from a clinical point of view, a specific PARP1 inhibitor – as opposed to an unspecific PARP inhibitor – seems more desirable for the following reasons: in our study, *PARP1* deletion was sufficient to decrease aortic PARP activity and reduce atherogenesis in mice by about 50%. Thus, specific PARP1 inhibition should be sufficient to reproduce these effects in patients. In addition, specific inhibition is likely to induce less side effects.

In our study, we found that chronic PARP inhibition induces skin lesions in about a fifth of PJ34-treated *ApoE*$^{-/-}$ mice and in about a tenth of *ApoE*$^{-/-}$*PARP1*$^{-/-}$ mice. Similarly, the development of skin lesions has previously been reported to occur in a third of older *PARP1*-deficient mice.[4] These findings suggest a possible link between skin lesions and PARP inhibition or *PARP1* deficiency. No such side effects, however, have been reported with newer generations of PARP inhibitors used in clinical studies.[33,34]

We conclude that endogenous PARP1 enhances atherogenesis by increasing adhesion molecule expression with endothelial activation, enhancing plaque inflammation with macrophages and T-cells, and by inducing features of plaque vulnerability. Thus, inhibition of enzymatic PARP1 activity may represent a promising therapeutic target in atherosclerosis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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