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Influence of Aspirin on SR-BI expression in human carotid plaques

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Abstract

Objective—We recently showed that aspirin promotes scavenger receptor class-B type I (SR-BI) protein expression *in vitro* in primary human macrophages and *in vivo* in resident peritoneal macrophages of mice.

Methods—We compared SR-BI and CD68 expression in carotid atherosclerotic specimens from endarterectomized patients with (n=38) or without (n=19) low-dose aspirin medication (100mg/d) prior to endarterectomy.

Results and Conclusion—We found no differences concerning expression of CD68, indicating that aspirin did not influence macrophage content within atherosclerotic plaques. However, aspirin increased the expression of SR-BI protein in the analyzed specimens. In human THP-1-derived macrophages, induction of SR-BI protein by aspirin was abrogated by concomitant pharmacological inhibition of nuclear factor – kappa B (NF- κ B). In cultured primary macrophages from NF- κ B/p50 KO mice, aspirin was not able to influence SR-BI expression. The same lack of effect on SR-BI expression was observed *in vivo* in resident macrophages of NF- κ B/p50 KO mice orally treated with physiological doses of aspirin. We suggest that aspirin treatment might lead to enhanced expression of SR-BI in human plaque macrophages and that this effect is dependent on the presence of NF- κ B.

1. Introduction

Scavenger receptor class B type I (SR-BI) was the first high density lipoprotein (HDL) receptor to be discovered ¹. CD36 and lysosomal integral membrane protein-II analogue 1 (CLA-1) has been identified as the human homologue of SR-BI ^{2, 3}. SR-BI mediates high affinity binding of HDL facilitating bidirectional flux of cholesterol across the plasma membrane ^{4, 5} and was described to be significantly expressed in cells that are involved in reverse cholesterol transport (RCT) ⁶, i.e. atherosclerotic plaque macrophages ^{7, 8} and hepatocytes ^{1, 9}. A recent *in vivo* study performed in mice suggested SR-BI of bone marrow-derived cells to prevent the progression of advanced atherosclerosis, most likely by promoting the efflux of excess cholesterol to HDL ¹⁰. Acetylsalicylic-acid (aspirin) is an

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established widely used agent for the therapy of inflammatory diseases, as well as for the primary and secondary prevention of vascular events such as myocardial infarction or stroke^{11, 12}. These benefits have mostly been attributed to the platelet-inhibitory and anti-inflammatory effects of aspirin^{12, 13}. Additionally, we recently reported on a COX-independent effect of aspirin on SR-BI expression *in vitro* in differentiated human macrophages as well as *in vivo* in resident macrophages of mice¹⁴. We showed that this effect occurred on a post-transcriptional level and suggested that it might involve the nuclear transcription factor NF- κ B¹⁴. In the present study we investigated aspirin-mediated effects on SR-BI expression *in vivo* in humans, as well as the role of NF- κ B in this scenario.

2. Materials and Methods

2.1. Human THP-1 cells

THP-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated by standard procedures. Differentiation into macrophages was achieved in supplemented RPMI 1640 cell culture medium containing 100 nmol/l of phorbol 12-myristate 13-acetate (PMA) (Promega, Madison, WI, USA) for 72 h. After incubation of differentiated macrophages with either placebo, aspirin (Alexis, Gruenberg, Germany) and/or pyrrolidinedithiocarbamate (PDTC) (Sigma, St. Louis, MO, USA), total cellular proteins were extracted for Western blot experiments employing an established protocol¹⁴.

2.2. Mice and primary murine macrophages

Certified NF- κ B/p50 KO C57BL6 mice were obtained from Charles River Laboratories (Kisslegg, Germany)¹⁵. All procedures and care of animals were approved by the Austrian Animal Care and Use Committee.

For *in vitro* experiments, animals were sacrificed by cervical dislocation, resident peritoneal macrophages isolated by peritoneal lavage, and incubated with vehicle or aspirin for 40 hours.

For *in vivo* experiments, animals received either normal drinking water or drinking water containing 60 mg/l aspirin, which was replaced every other day. On a body scale-adjusted scale, this amount would be equal to 360 to 540 mg/day if the animals weighed 60 kg¹⁴. After 7 days of treatment, animals were sacrificed by cervical dislocation and resident peritoneal macrophages were isolated by peritoneal lavage.

For both, *in vitro* and *in vivo* experiments, total cellular proteins from macrophages were finally extracted for Western blot experiments employing an established protocol¹⁴.

2.3. Patients and plaque specimens

Carotid plaque specimens consisting of atheromatous plaque, together with adjacent intima and medial layers, were collected from 57 consecutive patients with either symptomatic internal carotid artery stenosis of > 70 % or asymptomatic internal carotid stenosis > 90 % undergoing carotid endarterectomy (CEA). Degree of carotid stenosis was determined by colour duplex sonography at the Department of Neurology in Innsbruck. Symptomatic carotid disease was diagnosed in 33 patients with a history of transient ischemic attack, amaurosis fugax, hemiparesis or stroke, whereas 24 patients had asymptomatic carotid artery stenosis. According to the presence or absence of a medical treatment with acetylsalicylic acid (aspirin), patients were split into two groups: one group (n = 38) with a history of > 3 month of an anti-thrombotic treatment with aspirin (100 mg/d) and one group (n = 19) with a history of at least 3 months of anti-thrombotic treatment with clopidogrel (n=1), or without any anti-thrombotic treatment (n=18).

All patients were scheduled for elective therapeutic CEA and gave written informed consent to the surgical procedure and to the experimental use of the excavated carotid plaque tissue. After excision, carotid tissue specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C . Patient characteristics were retrieved from hospital records. This retrospective cross-sectional study was in accordance with the guidelines of the ethics committee of the Medical University of Innsbruck.

2.4. RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from approximately 100 mg of pulverized frozen carotid plaque tissue employing an established protocol with RNA-Bee™ (Tel-Test, Inc., Friendswood, Texas, USA) and reverse transcribed into cDNA with Omniscript-RT Kit (Qiagen, Hilden, Germany) (16). Primers and Taqman probe for human SR-BI have been described previously¹⁶. Forward and reverse primers and Taqman probes for the detection of human CD68 were designed with the Primer Express® software v.1.0 as follows: 5'-CAT TCC CCT ATG GAC ACC TCA-3' (forward), 5'-CCG CCA TGT AGC TCA GGT AGA-3' (reverse), and 5'-TTC ATG CAG GAC CTC CAG CAG AAG G-3' (probe). The modulation of SR-BI and CD68 was investigated by quantitative Real Time Taqman PCR using the Mx4000® Multiplex Quantitative PCR System (Stratagene, Amsterdam, The Netherlands) according to the user's manual.

18S rRNA, which was detected in all 57 samples, was used as reference gene (Applied Biosystems, Foster City, CA, USA). Corresponding relative expression values from SR-BI and CD68 mRNA were calculated according to the $\Delta\Delta\text{Ct}$ -method¹⁷.

2.5. Protein extraction and Western blot analysis

Total cellular proteins were extracted from approximately 150 mg of pulverized frozen carotid plaque tissue employing an established protocol¹⁴. Western blot analysis was performed as described previously¹⁸. Immunodetection of SR-BI was carried out with a monoclonal murine antibody against CLA-1 (BD Biosciences, Franklin Lakes, NJ, USA). For immunodetection of CD68 we employed a monoclonal murine antibody from Dako (Glostrup, Denmark). A horseradish-peroxidase-conjugated goat anti-mouse IgG antibody (Pierce Biotechnology Inc., Rockford, IL, USA) was used as secondary antibody. Immunodetection of β -actin (with a monoclonal anti- β -actin antibody from Sigma, St. Louis, MO, USA) was performed after stripping with Re-Blot Plus Mild Solution (Chemicon, Temecula, CA, USA). The chemoluminescent reaction was performed using Super Signal West Dura Reagent (Pierce Biotechnology Inc., Rockford, IL, USA) and blots were visualized and quantified by a Fluor-S-Imager using the Quantity One® v. 4.1.1 software (BioRad, Hercules, CA, USA). SR-BI and CD68 values from all specimens were adjusted to corresponding β -actin values.

2.6. Statistics

Mann Whitney *U*-test (MW *U*-Test), or χ^2 -Test (Fisher's Exact Test) where applicable, were used to analyze patient characteristics. MW *U*-Test was applied to compare independent not normally-distributed values from relative protein and mRNA data between plaque specimens from patients with and without aspirin treatment. Statistical analyses were performed using SPSS® software v11.0.

3. Results

3.1. Effect of aspirin on SR-BI in human carotid plaques

We analyzed carotid atherosclerotic specimens from endarterectomized patients with (n=38) or without (n=19) low-dose aspirin medication for at least three months prior to our

investigations. Statistical comparison of demographic data and of cardiovascular risk factors between the aspirin- and non aspirin-treated patients is shown in Table 1. Information concerning prevalence of coronary artery disease (CAD) and peripheral artery disease (PAD), as well as further medical treatment in both groups is shown in the same table. There were no significant differences in the demographic characteristics between the two groups of patients with or without aspirin. Furthermore, use of anti-diabetics, anti-hypertensives, and cholesterol-lowering statins were similar in both groups (Table 1).

Aspirin has been shown to be associated with decreased content of macrophages within atherosclerotic lesions¹⁹. We, therefore, measured expression of CD68, one of the most widely accepted antigens characterizing human tissue macrophages and foam cells²⁰. We did not observe significant differences in CD68-specific mRNA expression between carotid specimens from patients with or without aspirin treatment (data not shown). This finding was confirmed in subsequent measurement of CD68 expression on the protein level (Fig. 1). Thus, aspirin had no effect on macrophage content of carotid plaques in this clinical setting.

In previous *in vitro* experiments, we have described an influence of aspirin on the protein expression of SR-BI in primary human macrophages¹⁴. In the present *in vivo* study, we therefore examined the influence of aspirin on the expression of SR-BI in human carotid lesions. SR-BI mRNA was not significantly influenced by aspirin treatment (data not shown). However, on the protein level, aspirin treatment was associated with significantly increased SR-BI expression (Fig. 2).

3.2. Role of NF- κ B on aspirin-mediated SR-BI upregulation

A potential involvement of NF- κ B in the upregulating effect of aspirin on SR-BI has been proposed by us earlier¹⁴. Here, we show that the effect of aspirin is crucially depending on the presence of NF- κ B. In human THP-1-derived macrophages, increasing doses of the specific NF- κ B inhibitor PDTC up to 200 μ mol/l did not influence basal SR-BI expression (Fig. 3, left 3 lanes). However, PDTC suppressed the effect of aspirin on SR-BI in a dose-dependent manner (Fig. 3, right 3 lanes).

To confirm data from our NF- κ B inhibition experiments, we investigated the influence of aspirin on SR-BI expression in macrophages with non-functional NF- κ B. We therefore isolated resident peritoneal macrophages from NF- κ B/p50 KO mice and incubated them with increasing doses of aspirin up to 5 mmol/l. No effect of aspirin on SR-BI was observed (Fig. 4). These experiments indicated transcription factor NF- κ B to be required for the aspirin-associated enhancement of SR-BI expression in macrophages.

To test our *in vitro* findings also *in vivo*, male NF- κ B/p50 KO mice were randomized to receive vehicle or low-dose aspirin (60mg/l) in their drinking water, respectively. Based on the daily water intake of 2-3 ml for each mouse, the estimated daily intake of aspirin was calculated to be 6-9 mg/kg¹⁴. No apparent differences concerning mobility, behaviour, or food intake was observed during the entire study. After 1 week of treatment, resident peritoneal macrophages were isolated by peritoneal lavage. In contrast to our earlier studies employing wild-type mice¹⁴, we did not observe any effect of aspirin on SR-BI protein expression in macrophages of NF- κ B/p50 KO mice (Fig. 5).

4. Discussion

Van Berkel and coworkers have recently suggested an atheroprotective role for SR-BI in advanced atherosclerotic lesions¹⁰ which is believed to be due to an enhancement of net cholesterol efflux from lesion macrophages onto HDL. Recently, we described a positive influence of aspirin on SR-BI expression and function both, *in vitro* in primary human

macrophages and *in vivo* in resident murine macrophages after treatment of mice with aspirin in their drinking water¹⁴. In the present study we observed an increase of SR-BI expression within carotid lesions from patients treated with aspirin, exclusively on the protein level. Since SR-BI has been described to underlie post-transcriptional regulation, e.g. in response to PPAR- α ligands²¹, this finding was not unexpected. In fact, in our previous aspirin studies we had demonstrated for the first time that SR-BI can be regulated on a post-transcriptional level also in macrophages¹⁴. However, due to absence of significant PDZK1 expression in macrophages ([I.T., A.W., A.R.], unpublished data, 2007) – the protein conferring post-transcriptional regulation of SR-BI in polarized cells^{22, 23} – this regulation may be conferred by a not yet described player.

We have earlier described enhanced DNA-binding capacity of NF- κ B in primary human macrophages treated with aspirin¹⁴. Our present *in vitro* and *in vivo* experiments using NF- κ B/p50 KO mice demonstrated that aspirin necessitates NF- κ B to confer its effect on SR-BI protein expression in macrophages. However, basal expression of SR-BI was shown to be independent from the presence of this transcription factor. Together with the fact that no response element for NF- κ B was found within the SR-BI promoter² our data suggest, that NF- κ B does not directly influence transcriptional regulation of SR-BI, but rather is involved in regulation of another regulatory factor influencing transcriptional or posttranscriptional regulation of SR-BI.

Pharmacological substances other than aspirin, i.e. hypolipidemic statins and anti-diabetic glitazones, have been described to influence macrophage SR-BI expression^{24, 25}. However, in our study we did not observe any effect of these compounds on the expression of SR-BI in carotid lesions, neither on the transcriptional nor on the protein level (data not shown).

A weakness of this study may be the limited number of specimens. However, our sample size is equal or even greater compared to similar studies with endarterectomized carotid samples^{26, 27}. The apparent wider distribution of protein expression data found for aspirin-treatment may be explained by the higher portion of patients in that group. Different distribution ranges are most likely not the result of the methodological approach, as all carotid samples originate from the same surgical department and were equally processed and analyzed following an approved protocol.

Taken together, we were able to show that aspirin treatment is associated with increased SR-BI expression in human carotid lesions; we suggest this effect to be dependent on the presence of transcription factor NF- κ B. We believe that aspirin is indeed a SR-BI inducing agent not only *in vitro* in primary human macrophages and not only *in vivo* in mice, but also *in vivo* in humans. Our findings suggest an additional anti-atherogenic effect of aspirin on advanced atherosclerotic lesions, due to its effect on SR-BI regulation and, consequently, on reverse cholesterol transport.

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Fig. 1.

Expression of CD68 in carotid lesions from patients without and with aspirin treatment. Western blot signals were quantified employing Quantity-One® software. Relative expression of CD68 was calculated using corresponding β -actin signals from reblot analysis. Data from single specimens (open circles) and medians (bars) are shown. Statistical comparison to determine P -values was calculated with MW U -Test.

Fig. 2. Expression of SR-BI in carotid lesions from patients without and with aspirin treatment. Western blot signals were quantified employing Quantity-One® software. Relative expression of SR-BI was calculated using corresponding β -actin signals from reblot analysis. Data from single specimens (open circles) and medians (bars) are shown. Statistical comparison to determine *P*-values was calculated with MW *U*-Test.

Fig. 3.

Influence of PDTC – a specific inhibitor of transcription factor NF- κ B – on aspirin-mediated expression of SR-BI in THP-1-derived macrophages. A representative Western blot is shown for three independent experiments. β -actin served as reference.

Fig. 4. Influence of aspirin on SR-BI expression in cultured primary macrophages of NF- κ B/p50 KO mice. Macrophages were isolated by peritoneal lavage, seeded into 6-well plates and treated with indicated amounts of aspirin for 40 hours. A representative Western blot is shown for three independent experiments. β -actin served as reference.

Fig. 5.

Influence of aspirin on SR-BI expression in resident peritoneal macrophages of NF- κ B/p50 KO mice after 7 days of oral treatment with indicated amounts of aspirin. Each band of the displayed Western blot represents protein extracts pooled from 3 mice. β -actin served as reference.

Table 1

Patient characteristics

<i>Characteristics</i>	no aspirin (n=19)	aspirin (n=38)	<i>P</i>[‡]
<i>Age (mean ± SD) [y]</i>	69.6 ± 9.3	65.5 ± 9.6	0.102
<i>Males</i> *	68.4 (13)	79.0 (30)	0.516
<i>Degree of carotid stenosis [mean %]</i>	90.3 ± 3.1	89.1 ± 5.4	0.405
<i>Cardiovascular risk factors</i> *			
Smoking	47.4 (9)	55.3 (21)	0.589
Diabetes mellitus I + II	21.1 (4)	21.1 (8)	1.000
Hypertension	78.9 (15)	84.2 (32)	0.717
Hyperlipidemia	63.2 (12)	63.2 (24)	1.000
<i>Clinical diagnoses</i> *			
Symptomatic carotid disease [‡]	73.7 (14)	50.0 (19)	0.154
CAD	47.4 (9)	50.0 (19)	1.000
PAD	31.6 (6)	31.6 (12)	1.000
<i>Drugs</i> *			
Coumarins	21.1 (4)	2.6 (1)	0.038
Antidiabetics	21.1 (4)	13.2 (5)	0.463
Antihypertensives	63.2 (12)	76.3 (29)	0.356
Statins	26.3 (5)	44.7 (17)	0.251

[‡]*P*-values as determined by MW *U*-Test or χ^2 -Test (Fisher's Exact Test), where applicable

* Percentage of patients, total number of patients in brackets

[‡] History of transient ischemic attack, amaurosis fugax, hemiparesis or stroke

Abbreviations: CAD, coronary artery disease; PAD, peripheral artery disease.