Introduction

Hyaluronic acid (HA) is an unbranched, unsulfated carbohydrate component of the extracellular matrix that is synthesized at the plasma membrane by three HA-synthase isoforms (HAS1,-2,-3). HA mediates cellular signalling via HA-receptors such as RHAMM, CD44, LYVE-1 and toll-like receptors [1]. During the last decade, strong evidence evolved that HA is a key regulator of tumour growth and metastasis [2]. In arterial blood vessels, the expression of HA is mainly confined to the adventitia and the endothelial glyocalyx. During atherosclerosis, atherothrombosis and restenosis, HA is strongly induced and associates with proliferation of vascular smooth muscle cells (VSMC), neointimal expansion and possibly inflammation [3, 4]. HA is therefore thought to promote atherogenesis and neointimal hyperplasia [5]. Although many factors have been shown to stimulate HA synthesis in vitro [6, 7], little is known about the key factors regulating HA-accumulation in vivo and effects of drugs on cardiovascular HA-accumulation have not been studied yet. With respect to the specific functions of HAS-isoforms, it is known from HAS2-deficient mice that HAS2-mediated HA synthesis is critical for heart development and that deletion of HAS2 causes embryonic lethality [8]. In contrast, HAS1- and HAS3-deficient mice...
are viable. In adults, it is not known yet whether the three HAS-isofoms serve specific functions in the cardiovascular system and/or the pathophysiology of cardiovascular disease. We have recently observed that prostacyclin (PGI2) and prostaglandin E2 (PGE2) markedly induce HAS2 and HAS1 expression in cultured human VSMC [9, 10]. Cyclooxygenase 1 (COX-1) and COX-2 are constitutively expressed in endothelial cells, whereas COX-2 is strongly induced in VSMC by many of the major pro-atherogenic mediators such as PDGF-BB, cytokines, thrombin and oxidized LDL [11]. Therefore, we hypothesize that prostaglandins could indeed be key regulators of sustained neointimal HA-synthesis.

Because non-steroidal anti-inflammatory drugs (NSAID) that inhibit COX-dependent prostaglandin synthesis are widely used, this regulatory pathway might be of clinical relevance. Furthermore, in the light of the ongoing discussion about adverse cardiovascular effects of COX-2 inhibition, it will be important to consider also chronic effects on plaque remodelling [12]. Therefore, the role of COX products specifically in vascular HA synthesis was assessed in murine models of accelerated atherosclerosis and neointimal hyperplasia using the two prototypic non-isofom selective and COX-2-selective inhibitors, indomethacin and rofecoxib.

Materials and methods

Animals and experimental design

Male ApoE/− mice were obtained from Taconic M&B (Denmark) and kept on normal chow diet with or without 3 mg indomethacin or 50 mg rofecoxib per kg and day. Indomethacin from Sigma (Deisenhofen, Germany) and rofecoxib (Vioxx® tablets) were pelleted into the chow. ApoE-deficient mice were used in two disease models. First, HA-synthesis in atherosclerotic lesions was analyzed in ApoE-deficient mice receiving indomethacin or rofecoxib from 15 weeks to 23 weeks of age on normal chow (Fig. 1A). Second, ApoE-deficient mice underwent ligation of the left common carotid artery [13] to induce neointimal hyperplasia. Following carotid artery ligation, these mice were fed a Western diet (21% butter fat and 0.15% cholesterol) [13] to induce neointimal hyperplasia. Following carotid artery ligation, these mice were fed a Western diet (21% butter fat and 0.15% cholesterol) with or without the COX inhibitors for 4 weeks (Fig. 1B). All experiments were performed according to the guidelines for the use of experimental animals as given by the Deutsches Tierschutzgesetz and the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Urinary prostaglandin metabolites

Circulating PGI2 is rapidly converted into 2,3-dinor-6-keto PGF1α and TXA2 is rapidly hydrolyzed to form TxB2. The predominant urinary metabolite in mice is the β oxidation product 2,3-dinor TxB2. TxB2 and 2,3-dinor-6-keto PGF1α were measured by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS).

Urine of the mice was collected over a period of 48 hrs. A 200 μL sample of urine was spiked with 4 ng of d9-2,3-dinor-6-keto PGF1α and 2 ng of 13C8-2,3-dinor TxB2 and the methoximine derivative was formed by addition of 125 mg methoxamine hydrochloride in water (30 min., RT). The sample was purified by solid phase extraction (SPE) cartridge (StrataX SB-S100-TAK, Phenomenex, Torrance, CA). The chromatography was performed on a Hypersil 3 μm C18 (150 × 2.0 mm) analytical column (Phenomenex) using water and acetonitrile/methanol (95:5) as the mobile phase. A gradient of 10–38% mobile phase B was run over 30 min. at a flow rate of 0.200 ml/min. (Quantum Ultra, ThermoFinnigan, San Jose, CA) was performed using negative atmospheric pressure ionization and multiple reaction monitoring of the ion transitions m/z 370→155 (2,3-dinor TxB2), m/z 374→155 (13C8-2,3-dinor TxB2), m/z 370→232 (2,3-dinor-6-keto PGF1α) and m/z 373→235 (d9-2,3-dinor-6-keto PGF1α). Quantification of the endogenous metabolites used the ratio of the peak areas of the analyte and internal standard. Data were corrected for urinary creatinine (Oxford Biomedical Research, Oxford, MI).

RNA isolation

The total thoracic and abdominal aorta was separated from adventitia and frozen in liquid nitrogen. The whole common carotid artery was dissected between the aortic arch and the suture 4 weeks after ligation and also separated from adhering tissue. Total RNA was prepared using TRI-Reagent (Sigma). The RNA was quantified by spectroscopic analysis at 260 nm.

Characterization of mRNA expression

The expression levels were analyzed by quantitative real-time RT-PCR. Aliquots of total RNA (1000 ng) were applied for cDNA-synthesis using Superscript-III First-Strand synthesis system for RT-PCR (Invitrogen, Karlsruhe, Germany). Real-time RT-PCR analysis of mouse HAS1–3 and GAPDH was performed as described previously [14].

Immunohistochemical analysis

Whole hearts were fixed in Lillie’s neutral buffered 4% formaline solution and 5 μm paraffin sections of the aortic root were prepared for immunohistochemical stainings. Sections were stained for HA with biotinylated HA binding protein (2 ng/μl; Seikagaku, Tokyo, Japan) and HRP-conjugated streptavidin (1:200, Daco, Hamburg, Germany). SMC were detected with a monoclonal mouse anti-α-SM-actin clone 1A4 (1:1000, Sigma) and macrophages by an antibody against mac2 (1:400, Cedarlane, Burlington, Canada) and a rhodamine red-X-conjugated goat anti-rat IgG (preabsorbed to rodent, 1:400, Jackson ImmunoResearch, Suffolk, UK) used as a secondary antibody. Non-fluorescent detection was performed using dianamobenzidine (Zytomed, Berlin, Germany) as a chromogen.

Histochemical stainings

Three-micrometre paraffin sections of the aortic root were stained for collagen accumulation by picrosirius red. Qualitative analysis of collagen deposition was performed using polarized light microscopy.

Image analysis

Bright-field images were taken using a ColorView II and AnalySIS 3.2 software (Soft Imaging System, Münster, Germany) and analyzed by ImageJ 1.37v software (NIH) using the colour deconvolution technique and threshold values as described earlier [14].
Statistical analysis

Data are presented as the mean ± S.E.M. Statistical analysis was performed by unpaired t-test or one-way ANOVA followed by comparison of selected pairs (Bonferroni). A value of $P < 0.05$ was considered significant.

Results

HA-accumulation in atherosclerotic plaques

Mass spectrometric quantitation of urinary thromboxane A₂ (TxA₂) metabolite (2,3-dinor-TxB₂), an index of platelet COX-1 activity, revealed complete depression by indomethacin ($P < 0.05$) and no effect of rofecoxib (Fig. 1C). PGŁ₂ biosynthesis as assessed by quantitation of its urinary metabolite, 2,3-dinor-6-keto-PGF₁α, was depressed by >90% by indomethacin ($P < 0.05$) and by 75% by rofecoxib ($P < 0.05$). Roughly, 70% of PGŁ₂ formation is COX-2 dependent in mice [15]. Thus, rofecoxib acted, indeed, as selective inhibitor of COX-2 at our dosing regimen, whereas indomethacin inhibited – as expected – both isoenzymes (Fig. 1C). The overall condition of mice, including body weight and plasma levels of IL6, MCP1 and hsCRP, was not affected by indomethacin or rofecoxib (data not shown).

Plaque size at the aortic root was not changed by treatment with rofecoxib and indomethacin (not shown). However, treatment with both rofecoxib and with indomethacin resulted in decreased levels of HA in atherosclerotic plaques as determined by affinity histochemistry (Fig. 2A–D). Quantitative real-time RT-PCR (qRT-PCR) revealed significant inhibition of HAS1 mRNA and HAS2 mRNA expression in the thoracic aorta by indomethacin and rofecoxib (Fig. 2E). Furthermore, although HAS3 expression is not responsive to prostaglandins, a trend towards reduced mRNA expression was observed in response to both COX inhibitors. The reduction of HA-accumulation, HAS1 and HAS2 expression was paralleled by changes in the cellular composition of plaques. Alpha-SM-positive SMC in the caps of aortic root lesions were increased and mac2-positive macrophages were significantly decreased after indomethacin but not rofecoxib treatment (Fig. 3), as would be expected with COX-1 inhibition [16].

To investigate whether other matrix molecules were regulated as well by inhibition of COX-1 and COX-2 collagen accumulation was analyzed by picrosirius red staining. In aortic root plaques, collagen abundance was not affected by the treatment. As an indication for the arrangement and packing of collagen fibrils the picrosirius red staining was also analyzed and quantified by birefringence analysis under polarized light (Fig. 4). However, arrangement of collagen was also not changed in response to COX inhibitors, suggesting that the observed changes in HA-accumulation and HAS-isoform expression are indeed specific responses and not a general effect on matrix remodelling.

HA-accumulation after ligation injury

Rapid induction of neointimal hyperplasia occurs in ApoE-deficient mice after ligation of the carotid artery [13]. In this model, neointimal hyperplasia is driven mainly by SMC proliferation and migration and was used in comparison with the chronic model of atherogenesis as detailed earlier. Neointimal hyperplasia was significantly reduced by indomethacin that corresponded to decreased frequency of thrombotic occlusion of ligated carotid arteries (not shown). Arteries with thrombus formation were excluded from further analysis. In contrast, rofecoxib had no effect on neointima formation (not shown).

HA-accumulation was detected in the luminal part of the neointima and found to be strongly reduced by treatment with rofecoxib and indomethacin (Fig. 5A–D). Furthermore, only HAS1 mRNA expression was dramatically reduced by both COX inhibitors (Fig. 5E), whereas HAS2 was not changed. Moreover, indomethacin resulted in increased staining of alpha SM-actin in neointimal hyperplasia (Fig. 5F–I) similar to the effects observed in atherosclerotic plaques.
**Fig. 2** Aortic root lesions of ApoE-deficient mice stained for HA (A–D). (A) Control; (B) indomethacin (8 weeks, 3 mg/kg/day); (C) rofecoxib (8 weeks, 50 mg/kg/day); (D) densitometric quantitation of HA affinity histochemistry; (E) aortic mRNA expression of HAS isoforms; n = 15–12, mean ± S.E.M., *P < 0.05.

**Fig. 3** VSMC (α-SM actin, A–D) and macrophage (mac2, E–H) content of aortic root lesions of ApoE-deficient mice after treatment with COX inhibitors. A, E, controls; B, F indomethacin (8 weeks, 3 mg/kg/day); C, G, rofecoxib (8 weeks, 50 mg/kg/day); densitometric quantitation of D, α-SM actin and H, mac2; n = 12, mean ± S.E.M., *P < 0.05.
Fig. 4 Collagen content of aortic root lesions of ApoE-deficient mice indicated by picrosirius red staining after treatment with COX inhibitors. Total collagen was evaluated by light microscopy (A–D) and collagen arrangement was analyzed by birefringence analysis using polarized light (E–H). A, control; B, indomethacin (8 weeks, 3 mg/kg/day); C, rofecoxib (8 weeks, 50 mg/kg/day); densitometric quantitation of D, picrosirius red staining, light microscopy and H, picrosirius red staining under polarized light to visualize densely packed collagen (bright red); n = 12, mean ± S.E.M., *P < 0.05.

Fig. 5 HA-accumulation in neointimal hyperplasia 4 weeks after ligation of the left carotid artery of ApoE-deficient mice on a Western diet. (A) Control; (B) indomethacin (4 weeks, 3 mg/kg/day); (C) rofecoxib (4 weeks, 50 mg/kg/day); (D) densitometric quantitation of HA-affinity histochemistry in the neointima; (E) mRNA expression of HAS isoforms in ligated carotid arteries; (F–H) α-SM-actin staining; densitometric quantification of α-SM-actin; n = 4–7; mean ± S.E.M., *P < 0.05.
lesions (compare to Fig. 3). Macrophage accumulation as evidenced by immunohistochecmy of mac2 was not responsive to COX inhibition in the carotid injury model (Fig. 6). Similar to the results in atherosclerotic plaques, the amount collagen deposited in the neointima was not affected (not shown).

Discussion

The biology of the COXs in vascular lesions is not well understood. Multiple cell types such as macrophages, VSMC and endothelial cells expressing COX-1, COX-2 or both interact during the development of atherosclerosis. These cell types are thought to have distinct prostaglandin biosynthesis profiles depending on the presence of, for example, prostaglandin synthase, thromboxane A2 synthase and prostacyclin synthase. Furthermore, the response to these prostanoids is dependent on the prostanoid receptor subtype expression in the respective cell types. Although COX-1-derived TxA2 accelerates atherogenesis in mice [17], COX-2 deletion or inhibition may accelerate [18], retard [19] or leave lesion progression unaltered [20] in mice. These conflicting observations may perhaps be explained by the contrasting cardiovascular effects of COX-2 products derived from the distinct cellular sources as detailed earlier and their relative importance in distinct phases of the disease. The specific aim of the present study was to evaluate whether COX inhibition impacts the biology of HA synthesis and deposition in the neointima; n = 4–7; mean ± S.E.M., *P < 0.05.

![Fig. 6](image)

Macrophage staining (mac2) in neointimal tissue 4 weeks after ligation of the left internal carotid artery in ApoE-deficient mice on Western diet. (A) Control; (B) indomethacin (4 weeks, 3 mg/kg/day); (C) rofecoxib (4 weeks, 50 mg/kg/day); (D) densitometric quantitation of mac2 within the neointima; n = 4–7; mean ± S.E.M., *P < 0.05.

The present findings support strongly the conclusion that chronic administration of COX inhibitors interfere with ECM remodelling and specifically with HA matrix synthesis and accumulation in atherosclerotic lesions. These effects on HA might...
affect progression and stability of atherosclerotic lesions and should be considered as a potential additional facet of chronic COX inhibitor treatment. Clearly, further studies are needed to address the roles of HAS1/-2 and the COXs during atherosclerosis in detail. This seems particularly important given that a large-scale trial to address potential cardioprotective effects of a COX-2 inhibitor is ongoing – the Prospective Randomized Evaluation of Celecoxib Integrated Safety vs. Ibuprofen or Naproxen (PRECISION) (ClinicalTrials.gov Identifier: NCT00346216).

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