Nitric oxide synthesis is involved in arterial haptoglobin expression after sustained flow changes

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Abstract The acute phase protein haptoglobin is highly expressed in arteries after sustained flow changes and involved in cell migration and arterial restructuring. In the liver, haptoglobin expression is mainly regulated by interleukin-6 (IL-6). In the artery, shear stress and NO influence IL-6 expression. In the present study, we demonstrate that NO synthesis is involved in the regulation of arterial haptoglobin expression after sustained flow changes. Decreased haptoglobin expression after NO inhibition coincided with decreased IL-6 levels. However, IL-6 knockout mice had normal arterial haptoglobin expression levels after sustained flow changes suggesting that other mediators may provide compensatory mechanisms for the regulation of arterial haptoglobin expression.

Key words: Arterial remodeling; Nitric oxide; Haptoglobin; Flow change

1. Introduction

Chronic changes in blood flow induce structural remodeling of the arterial wall to normalize shear stress [1]. Structural remodeling is an important determinant of luminal narrowing after balloon angioplasty and in de novo atherosclerosis. Previously, we demonstrated that arterial expression of the acute phase protein haptoglobin is increased during arterial remodeling after sustained flow changes and plays an important role in cell migration and arterial restructuring [2]. However, the regulatory pathways involved in arterial haptoglobin expression are unknown.

Liver haptoglobin expression is thought to be mediated through interleukin-6 (IL-6), rather than other inflammatory cytokines like glucocorticoids or tumor necrosis factor α (TNFα). This is supported by point mutations in IL-6 responsive elements in the haptoglobin promoter region, resulting in lower serum haptoglobin levels [3,4]. Arterial IL-6 expression has been demonstrated in endothelial and smooth muscle cells during atherosclerosis [5,6], pointing to a regulatory role in arterial haptoglobin expression.

IL-6 expression in endothelial cells can be regulated by shear stress [7] and nitric oxide (NO) [8]. NO is an important mediator of arterial remodeling as is demonstrated by studies using either NO synthase (NOS) inhibitors [9] or endothelial NOS (eNOS) [10] and inducible NOS (iNOS) [11] knockout mice. NO mediates different processes that lead to arterial remodeling which involves the modulation of metalloproteinase (MMP) expression and activity [12] and cell migration [13]. Previous studies have shown that MMP inhibition can prevent constrictive remodeling and subsequent luminal narrowing [14–17].

We hypothesized that NO production after arterial flow changes regulates arterial expression of haptoglobin, a gelatinase inhibitor, through IL-6 expression. In this study, we show that NO synthesis is involved in the regulation of arterial haptoglobin expression after sustained flow changes and suggest that this NO–haptoglobin pathway is an important cellular mediator between sustained flow changes and arterial restructuring.

2. Materials and methods

2.1. Animal models

The investigation conforms with 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).

2.1.1. Rabbits. In 14 New Zealand White rabbits, the right carotid artery was completely ligated which also resulted in flow increase in the contralateral left carotid artery. Rabbits received either 1.5 g/l L-NAME in the drinking water, starting 5 days before intervention (L-NAME-treated group, $n=7$) or normal water (control group, $n=7$). The carotid arteries of six additional unoperated rabbits were used to obtain baseline expression levels. The rabbits were anesthetized by intramuscular injection of methadone (0.15 ml) and Ventraquil (0.15 ml) followed by intravenous injection of etomidate (1 mg/kg) and ventilated with N_2O:O_2 and 0.6% halothane. Animals were killed 1 day after operation and the carotid, femoral and iliac arteries were removed. The arteries were snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction.

2.1.2. Mice. The IL-6 knockout mice had been backcrossed into the wild-type B6/129 background for at least 15 generations at the time of the experiments. Male wild type and IL-6 knockout mice ($n=30$) were anesthetized using 0.05 ml/10 g body weight of a cocktail (1 part Hypnorm, 1 part midazolam, 2 parts distilled water). The right common carotid artery was ligated as described by Kumar and Lindner [18]. Animals were killed at 0 days ($n=5$/group) or 3 days ($n=10$/group) after operation and the left and right carotid arteries were removed. The arteries were snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction.

2.2. RNA extraction

The frozen tissue samples were ground in liquid nitrogen, using a pestle and mortar. RNA was extracted using 1 ml Tri-pure™ Isolation

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Reagent (Boehringer Mannheim) according to the manufacturer's protocol.

2.3. Northern blotting

Ten μg of RNA was separated on a 1.2% formaldehyde-agarose gel. After capillary blotting to Hybond-N membrane (Amersham), the membrane was baked for 2 h at 80°C. Probes were prepared using the random-primed DNA labeling kit (Boehringer Mannheim). Hybridization occurred for 1 h at 65°C in Easy-Hyb hybridization solution (Stratagene) followed by 10 min washing in 0.1×standard saline citrate, 0.1% sodium dodecyl sulfate at 65°C. Bands were visualized using Biomax MS films (Kodak).

2.4. Quantitative real-time PCR

Reverse transcription was carried out with 500 ng total RNA using Superscript II (Life) according to the manufacturer's protocol. To confirm the identity of the amplified cDNA products, PCR products were ligated into the pGEM®-T Easy Vector (Promega) and sequenced using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham).

PCR amplification was performed using the I-cycler iGTM Real Time PCR (Bio-Rad). Each reaction contained 14 μl cDNA, 200 μM dNTP, 1×reaction buffer (Gibco BRL) containing 1:80 0000 Cybergreen (Bio-Rad), 2.5 U Taq DNA polymerase (Gibco BRL) and 1 μM of each primer. Quantities were determined by comparison with known quantities of the cloned PCR product representing the target mRNA. Data were corrected for the amount of 18S mRNA that was used as an internal standard.

The following oligonucleotides were used as primers: rabbit haptoglobin (forward 5′-GAAGCAGTGGGTGAACAAGG-3′, reverse 5′-TGACAAGATTGTGGCGGGAG-3′), rabbit interleukin-6 (forward primer 5′-ACCACGATCCACTTCATCC-3′, reverse primer 5′-TGTCCTAACGCTCATCTTC-3′), rabbit 18S (forward primer 5′-TCAACACGGGAAACCTCAC-3′, reverse primer 5′-ACAAATCGCTCCAGCAAC-3′), mouse haptoglobin (forward 5′-AAAAACCTCTTCCTGAACCAC-3′, reverse 5′-AACGACCTTCTCAATCTCCAC-3′), mouse 18S (forward 5′-TCAACACGGGAAACCTCAC-3′, reverse 5′-ACCAGACAAATCGCTCCAC-3′).

2.5. Statistics

Data are presented as mean ± S.E.M. Statistical analysis was performed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

3. Results

To investigate the role of NO in the regulation of arterial haptoglobin expression after flow changes in rabbit carotid arteries, NO synthesis was inhibited using the non-specific NOS inhibitor L-NAME. Northern blot analysis showed that after flow changes, haptoglobin mRNA levels increased more in carotid arteries of control rabbits compared to the carotid arteries of L-NAME-treated rabbits, 1 day after flow changes (Fig. 1A).

Quantitative real-time PCR was used to quantify the relative amounts of haptoglobin mRNA levels in the carotid arteries of unoperated, control and L-NAME-treated rabbits. In control rabbits, both a flow increase and a flow decrease in the carotid artery resulted in a significant increase of arterial haptoglobin mRNA expression compared to baseline values (30-fold, $P = 0.005$; Fig. 1B). In L-NAME-treated rabbits, a significant increase in haptoglobin mRNA levels was observed compared to baseline values (12-fold, $P = 0.005$). Absolute haptoglobin mRNA levels, however, were significantly decreased in the carotid arteries of L-NAME-treated rabbits compared to control rabbits (33%, $P = 0.04$; Fig. 1B). Basal haptoglobin mRNA expression in unoperated femoral and iliac arteries was not affected by L-NAME treatment (Fig. 1C).

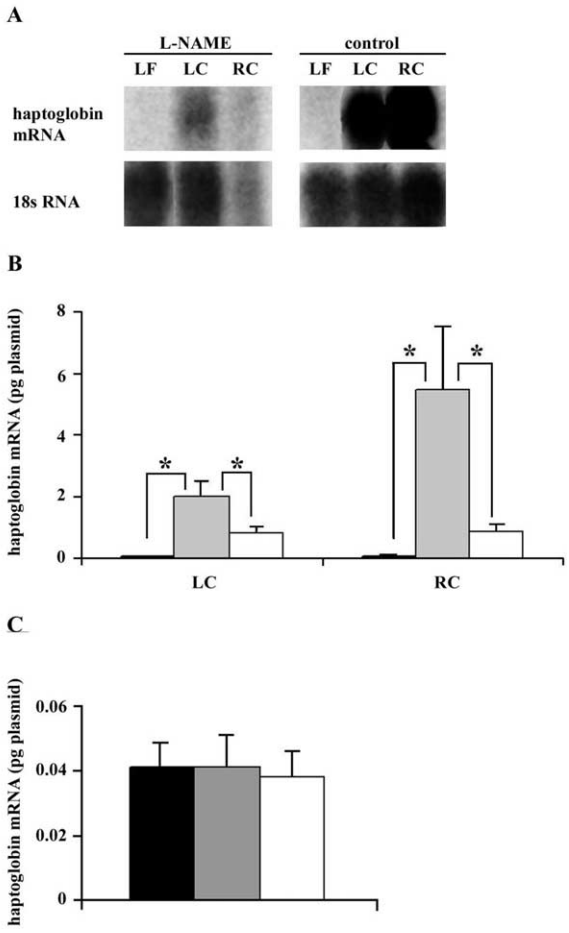

Fig. 1. Arterial haptoglobin mRNA expression in rabbit carotid arteries after L-NAME treatment, 1 day after sustained flow changes. A: Northern blot hybridization showing haptoglobin mRNA expression in L-NAME-treated and control rabbits, 1 day after flow changes. LF = unoperated left femoral artery; LC = left carotid artery; RC = right carotid artery. B: Haptoglobin mRNA expression in carotid arteries after sustained flow changes. C: Haptoglobin mRNA expression in unoperated femoral and iliac arteries. Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product with which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. $n = 7$ rabbits/group, $^*P < 0.05$; black bar = baseline values; gray bar = control rabbits; white bar = L-NAME-treated rabbits.

IL-6 is thought to be the major stimulator of haptoglobin expression. Therefore, we investigated if IL-6 expression decreased after L-NAME treatment. One day after flow changes, IL-6 mRNA levels were significantly increased compared to baseline values in both the left and right carotid artery of control rabbits (four-fold, $P = 0.03$; Fig. 2A). However, no increase in IL-6 expression levels was observed in the carotid arteries of L-NAME-treated rabbits compared to baseline values. L-NAME treatment had no effect on basal expression levels of IL-6 mRNA in unoperated femoral and iliac arteries (Fig. 2B).

To confirm the possible role of IL-6 in arterial haptoglobin expression, haptoglobin expression was measured in IL-6 knockout mice following ligation of the right carotid artery (Fig. 3). In wild type mice, haptoglobin mRNA levels increased in the left and right carotid artery at 3 days after ligation of the right carotid artery (two-fold, $P = 0.05$). A similar increase in haptoglobin mRNA expression was observed

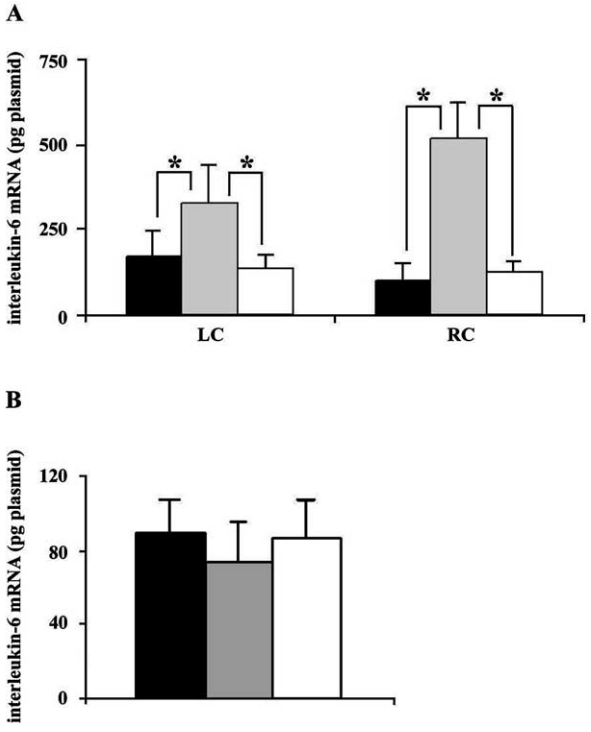

Fig. 2. IL-6 mRNA expression in rabbit carotid arteries after L-NAME treatment, 1 day after sustained flow changes. IL-6 mRNA is presented as the amount of plasmid containing the IL-6 PCR product with which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. A: IL-6 mRNA expression in carotid arteries after sustained flow changes. B: IL-6 mRNA expression in unoperated femoral and iliac arteries. $n = 7$ rabbits/group, $*P = 0.04$; LC = left carotid artery; RC = ligated right carotid artery; black bar = baseline values; gray bar = control rabbits; white bar = L-NAME-treated rabbits.

in IL-6 knockout mice 3 days after ligation of the carotid artery (two-fold, $P = 0.05$). Baseline haptoglobin expression levels did not differ between wild type and IL-6 knockout mice (Fig. 3).

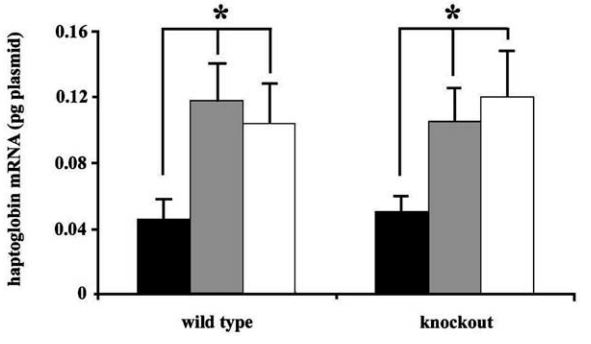

Fig. 3. Haptoglobin mRNA expression in carotid arteries in IL-6 knockout mice after sustained flow changes. Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product with which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. $n = 8$–10 arteries/group, $*P = 0.05$; black bar = carotid arteries, 0 days; gray bar = left carotid artery 3 days after ligation; white bar = ligated right carotid arteries, 3 days after ligation.

4. Discussion

Haptoglobin expression is increased in arteries after sustained flow changes and involved in cell migration and arterial restructuring [2]. The arterial response to normalize wall shear stress after blood flow changes depends on a functional vascular endothelium [19] that releases NO as a mediator in the arterial remodeling process. In the present study, we used the arterial ligation model to investigate if NO induces arterial haptoglobin expression after flow changes.

Inhibition of NO after flow changes using the non-specific NOS inhibitor L-NAME partly inhibited arterial haptoglobin expression. This shows that a NO-dependent pathway is involved in the regulation of arterial haptoglobin expression after flow changes. There are at least two isoforms of NOS expressed in the vascular system after injury or flow changes [20–22]. There are, however, differences in function between those isoforms. NO derived from eNOS inhibits neointimal formation whereas NO derived from iNOS promotes neointimal formation. Moreover, constrictive remodeling is impaired in eNOS knockout mice whereas iNOS knockout mice display more constrictive remodeling compared to wild type mice [10,11,23]. Unfortunately, the use of L-NAME did not allow for identification of the NOS isoform that is involved in the regulation of arterial haptoglobin expression.

There are no reports demonstrating a direct effect of NO or shear stress on haptoglobin expression. However, some of the genes which are regulated by NO appear to depend on the presence of diffusible intermediates like cytokines [24]. IL-6 expression is an important regulator of haptoglobin expression and expressed in both endothelial [7,8] and smooth muscle cells [5,6] during atherosclerosis. Moreover, IL-6 expression can be regulated by NO and shear stress [7,8], making it a potential candidate to function as an intermediate between increased NO synthesis and arterial haptoglobin expression.

To investigate a possible regulatory role for IL-6 in arterial haptoglobin expression, we measured IL-6 expression levels after L-NAME treatment. Although the L-NAME-treated rabbits still responded to flow changes with increased haptoglobin mRNA levels, there was a significant decrease in the absolute levels of haptoglobin mRNA. This decrease in haptoglobin coincided with baseline levels of IL-6, supporting a regulatory role for IL-6 in arterial haptoglobin expression.

IL-6 knockout mice were used to confirm if arterial haptoglobin expression is indeed regulated through IL-6 after sustained flow changes. Surprisingly, the IL-6 knockout mice responded normally to flow changes with increased haptoglobin mRNA levels, demonstrating that IL-6 is not the only regulator for the induction of haptoglobin in the arterial wall. This is in accordance with other studies showing that IL-6 is not the sole regulator of haptoglobin expression [25]. For instance, the induction of serum haptoglobin in IL-6 and IL-6/TNF/leukotriene α knockout mice is only slightly reduced after stimulation with lipopolysaccharide [26]. Apparently, multiple signals exist which are able to induce haptoglobin expression and can compensate for deficiencies in regulators like IL-6.

In summary, this study demonstrates that NO synthesis is involved in arterial expression of haptoglobin after sustained flow changes, coinciding with increased IL-6 levels. Since the increase in haptoglobin expression is normal in IL-6 knockout

mice after flow changes, we infer that other unidentified mediators may provide compensatory mechanisms for the regulation of arterial haptoglobin expression after flow changes.

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