Hepatocyte gp130 Deficiency Reduces Vascular Remodeling After Carotid Artery Ligation

Gustavo Salguero, Harald Schuett, Joanna Jagielska, René Schley, Ezequiel Tallone, Maren Luchtefeld, Helmut Drexler, Werner Müller, Karsten Grote, Bernhard Schieffer

Abstract—Inflammation and vascular remodeling are hallmarks of atherosclerosis, hypertension, and restenosis after angioplasty. Here we investigated the role of the hepatocyte gp130-dependent systemic acute phase response on vascular remodeling after carotid artery ligation. Mice with a hepatocyte-specific gp130 knockout on an apolipoprotein E/−/− background (gp130−/−) were compared with control mice (gp130flox). Vascular remodeling was induced by permanent ligation of the left common carotid artery. This, in turn, activated the systemic acute phase reaction in gp130flox mice, as measured by serum amyloid A plasma levels, which was completely abrogated in gp130−/− mice (P<0.05). Morphometric analysis of the carotid artery revealed severe neointima formation and media thickening 28 days after ligation in gp130flox mice, which was suppressed in gp130−/− mice (P<0.01). Serial sections from gp130−/− carotid segments showed significantly less smooth muscle cell (SMC) proliferation and monocyte recruitment (P<0.01). To evaluate the impact of the gp130-dependent systemic acute phase response on SMCs, hepatocytes from gp130flox and gp130−/− mice were stimulated with interleukin 6. Interleukin 6–induced secretion of serum amyloid A was completely abolished in gp130−/− hepatocytes (P<0.01). Moreover, when stimulated with supernatants from gp130−/− hepatocytes, SMCs showed significantly less migration and proliferation compared with supernatants from gp130flox hepatocytes (P<0.01). Recombinant serum amyloid A induced SMC migration and proliferation (P<0.05) and serum amyloid A injection after carotid artery ligation restored vascular remodeling in gp130−/− mice (P<0.01). These results imply a critical role for the gp130-dependent systemic acute phase response for vascular inflammation and SMC migration, as well as proliferation, and, subsequently, for vascular remodeling. (Hypertension. 2009;54:1035-1042.)

Key Words: vascular remodeling ■ gp130 ■ acute phase reaction ■ SMC migration/proliferation

Vascular remodeling is defined as any enduring change in size or composition of blood vessels in response to altered blood flow, mechanical load, or after vascular injury (eg, in atherosclerosis, hypertension, or restenosis after angioplasty).1,2 Neointima formation is one of the hallmarks of the remodeling process and involves cellular as well as structural changes of the vascular wall, which are driven by local infiltration of inflammatory cells and by migration and proliferation of resident medial smooth muscle cells (SMCs).3

The acute phase response is the immediate set of local and systemic inflammatory reactions triggered by tissue injury or infection to promote regenerative or adaptive processes.4 Members of the interleukin (IL) 6 cytokine family stimulate the systemic acute phase response via the gp130 receptor component in hepatocytes to secrete acute phase proteins, such as C-reactive protein and serum amyloid A (SAA), which are not only markers of inflammation but also powerful predictors of cardiovascular events.5,6 We reported recently that mice with a hepatocyte-specific deletion of the gp130 gene on an atherosclerosis-prone (apolipoprotein [apo] E/−/−) background showed reduced plasma levels of the predominant murine acute phase protein SAA,11 less macrophages within atherosclerotic plaques, and consequently less atherosclerosis.12 In this regard, SAA has been detected in atherosclerotic plaques and acts as a potent chemoattractant for macrophages and leukocytes.13,14

To outline the role of the systemic acute phase response for vascular remodeling, we subjected mice lacking hepatocyte-specific gp130 on an apoE/−/− background to an experimental model of vascular remodeling. Therefore, we ligated the left common carotid artery, which subsequently led to a profound neointima formation and media thickening proximal to the site of ligation.15 We observed a strong reduction in neointima hyperplasia and media thickening in hepatocyte-specific, gp130-deficient mice, which was associated with reduced local vascular inflammation, as well as reduced SMC content.
and proliferation in the vessel wall. Moreover, we found enhanced SMC migration and proliferation by recombinant SAA and SAA injection after carotid artery ligation restored vascular remodeling in gp130⁻/⁻ mice. In summary, our results suggest that the gp130-dependent systemic acute phase response is a critical regulator of local vascular remodeling processes.

**Methods**

**Animals**
Mice lacking the gp130 gene in the liver on an atherosclerosis-prone background were generated as described previously. In brief, albumin promoter–controlled expression of the Cre recombinase (alb-cre<sup>+</sup>) ensured a hepatocyte-specific deletion of the floxed gp130 gene (gp130<sup>flk/flox</sup>) on an apoE-deficient background (apoE<sup>−/−</sup>). The resulting genotype, alb-cre<sup>+</sup>;gp130<sup>flk/flox</sup>;apoE<sup>−/−</sup>, is simplified by gp130<sup>−/−</sup>. Littermates with a floxed gp130 gene on an apoE-deficient background (gp130<sup>flk/flox</sup>;apoE<sup>−/−</sup>) served as controls (gp130<sup>flk</sup>). Animals were backcrossed on a C57BL/6 genetic background and housed in the animal facility at the Hannover Medical School during experiments. All of the experiments were approved by the governmental animal ethics committee and performed according to the guidelines of the Federation of European Animal Science Associations.

**Carotid Artery Ligation**
As described by Kumar and Lindner, permanent ligation of the left common carotid artery was performed in 3- to 4-month-old male and female gp130<sup>−/−</sup> and gp130<sup>flk</sup> mice. The contralateral nonligated right carotid artery served as the control in all of the experiments (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org). Animals were anesthetized by IP injection of ketamine (400 mg/kg of body weight) and xylazine (5 mg/kg of body weight). The skin at the neck was opened by an incision along the midline, and the left common carotid artery was carefully exposed and ligated proximal to the carotid bifurcation using a 6-0 nylon silk. For some experiments, gp130<sup>−/−</sup> mice received a single IV injection of 300 µg of SAA in 200 µL of NaCl 30 minutes after carotid artery ligation. All of the animals recovered well and showed no symptoms of stroke. Animals were euthanized at 3, 7, and 28 days after carotid ligation. After excision of the left and right common carotid arteries, the vessels were embedded in OCT compound (Tissue-Tek), frozen on dry ice, and stored at −80°C for morphometry or immunohistochemistry.

**Results**

**Vascular Remodeling Induced by Carotid Artery Ligation Is Suppressed in gp130⁻/⁻ Mice**
Carotid artery ligation was performed in gp130⁻/⁻ and gp130<sup>flk</sup> control mice, as described recently by Kumar and Lindner. After 28 days, we observed a marked neointima formation of the ligated carotid artery in gp130<sup>flk</sup> mice compared with the nonligated artery, which was strongly reduced in gp130⁻/⁻ mice (78 473±25 414 versus 26 531±18 704 µm²; P<0.01; Figure 1A and 1B). Neointima formation in gp130<sup>flk</sup> mice after ligation was accompanied by enhanced media thickening, which was significantly reduced in gp130⁻/⁻ mice (132 367±46 529 versus 33 368±11 696 µm²; P<0.01; Figure 1A and 1C).

**SMC Content and Proliferation in the Vessel Wall Induced by Carotid Artery Ligation Is Suppressed in gp130⁻/⁻ Mice**
Because vascular remodeling after carotid artery ligation is thought to be driven by SMC proliferation, we evaluated the SMC content by immunostaining for &alpha-SMC actin. The increase in SMC content observed after carotid artery ligation in gp130<sup>flk</sup> mice was significantly reduced in gp130⁻/⁻ mice (51 899±6965 versus 16 217±4229 µm²; P<0.01; Figure 2A and 2B). We next analyzed cell proliferation in the carotid arteries by immunostaining for the proliferation marker Ki67. Ki67-positive nuclei in the ligated carotid artery of gp130<sup>flk</sup> mice already increased at day 3 after ligation peaked at day 7 and declined again at day 28. Cell proliferation in gp130⁻/⁻ mice was particularly inhibited at day 7 (8.02±0.84% versus 2.21±0.66%; P<0.01) after carotid artery ligation (Figure 2C and 2D). Proliferating cells in the carotid artery were identified as SMCs, because they were double positive for Ki67 and &alpha-SMC actin (data not shown).
Figure 2. SMC content and cell proliferation in the vessel wall induced by carotid artery ligation is suppressed in gp130−/− mice. A, Sections stained for α-SMC actin from the left and the right common arteries of gp130flox and gp130−/− mice 3 days after ligation. Representative pictures are shown (n=6). B, Quantification of the α-SMC actin positive area (n=6). *P<0.05 vs control, **P<0.01 vs control, ##P<0.01 vs gp130flox. C, Sections stained for the proliferation marker Ki67 from the left and the right common arteries of gp130flox and gp130−/− mice 3, 7, and 28 days after ligation. Representative pictures are shown (n=6). D, Quantification of Ki67-positive nuclei of total nuclei in the vessel wall. **P<0.01 vs control, #P<0.05 vs gp130flox, ##P<0.01 vs gp130flox.

Figure 3. Monocyte infiltration into the vessel wall induced by carotid artery ligation is suppressed in gp130−/− mice. A, Sections stained for MOMA-2 from the left and the right common arteries of gp130flox and gp130−/− mice 3 days after ligation. Representative pictures are shown (n=6). B, Quantification of the MOMA-2–positive area (n=6). *P<0.05 vs control, **P<0.01 vs control, ##P<0.01 vs gp130flox.

Monocyte Infiltration Into the Vessel Wall and Activation of the Systemic Acute Phase Response Induced by Carotid Artery Ligation Are Suppressed in gp130−/− Mice

We next evaluated the early local inflammatory response in ligated carotid arteries by immunostaining for MOMA-2. Compared with the nonligated carotid artery, we observed a strong enhancement of infiltrated monocytes in gp130flox mice 3 days after carotid ligation, which was inhibited in gp130−/− mice (4370±295 versus 1319±403 µm²; P<0.01; Figure 3A and 3B). To investigate the hepatocyte gp130-dependent activation of the systemic acute phase response, we determined plasma levels of the most dominant murine acute phase protein SAA.11 Carotid artery ligation remarkably increased acute phase SAA (SAA1) plasma levels in gp130−/− mice compared with nonligated controls at day 1 (158.7±30.3 versus 18.0±3.3 µg/mL; P<0.01) and returned to control levels thereafter (Figure 4A). In contrast, carotid artery ligation did not induce a significant increase in SAA plasma levels in gp130flox mice compared with nonligated controls at day 1 (158.7±30.3 versus 18.0±3.3 µg/mL; P<0.01) and returned to control levels thereafter (Figure 4B).
and 4C). Finally, we investigated SAA levels in the supernatants of cultured hepatocytes isolated from gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice. Hepatocytes from gp130\(^{\text{flox}}\) mice already secreted substantial amounts of SAA under basal conditions, which were found to be significantly induced when gp130 was stimulated with IL-6 (5.38±0.13 versus 1.76±0.38 \(\mu\)g/mL; \(P<0.01\)). However, hepatocytes from gp130\(^{-}\) mice secreted little SAA, even after IL-6 stimulation (Figure 4D). Of interest, using a different model of acute phase activation by lipopolysaccharide injection, we observed considerably higher plasma SAA levels (\(\approx 1500 \mu\)g/mL) but no differences between gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice (Figure S2).

### Acute Phase Protein SAA Induced SMC Migration and Proliferation and Restored Vascular Remodeling in gp130\(^{-}\) Mice After Carotid Artery Ligation

Systemic gp130-dependent acute phase response may critically influence vascular remodeling by local SMC migration and proliferation. To investigate this issue, we analyzed migration and proliferation of SMCs in vitro in response to the above-described IL-6–stimulated supernatants of hepatocytes isolated from gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice. SMCs were isolated as described recently,\(^{16}\) and SMC migration (as analyzed by transwell inserts) was found to be significantly induced in response to supernatants from gp130\(^{\text{flox}}\) hepatocytes but not in response to supernatants from gp130\(^{-}\) hepatocytes (42 250±5828 versus 21 750±3515 cells; \(P<0.01\); Figure 5A). In addition, we performed scratch assays with wild-type SMCs using the same supernatants. Similarly to transwell experiments, we observed enhanced wound closure in response to supernatants from gp130\(^{\text{flox}}\) hepatocytes as compared with control conditions, which was completely blocked in response to supernatants from gp130\(^{-}\) hepatocytes (1367±262 versus 183±231 \(\mu\)m\(^2\); \(P<0.05\); Figure 5B). Proliferation of wild-type SMCs was quantified by 5-bromodeoxyuridine (BrdUrd) incorporation and was found to be significantly increased in response to supernatants from gp130\(^{\text{flox}}\) hepatocytes and completely suppressed in response to supernatants from gp130\(^{-}\) hepatocytes (0.43±0.06 versus 0.18±0.04 optical density [OD]\(_{450}\); \(P<0.01\); Figure 5C).

Finally, we investigated whether hepatocyte-derived acute phase proteins themselves may be directly responsible for SMC migration and proliferation. We focused especially on the predominant murine acute phase protein SAA,\(^{12}\) which was increased in supernatants of gp130\(^{\text{flox}}\) hepatocytes in response to IL-6 (Figure 4D). Immunoprecipitation with antibodies against SAA significantly reduced SAA levels in response to IL-6–stimulated gp130\(^{\text{flox}}\) hepatocytes determined by trans-

---

**Figure 4.** SAA levels induced by carotid artery ligation in vivo and by IL-6 in vitro are inhibited in gp130\(^{-}\) mice. A, Plasma collected from gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice not ligated (control) and at days 1, 3, 7, and 28 after common carotid artery ligation was analyzed for SAA levels by ELISA (n=5). **\(P<0.01\) vs control, \#\(P<0.05\) vs gp130\(^{\text{flox}}\). B, Sections stained for SAA from the left and the right common arteries of gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice 3 days after ligation. Representative pictures are shown (n=5). C, **Quantification of the SAA-positive area (n=5). **\(P<0.01\) vs control, \#\(P<0.05\) vs gp130\(^{\text{flox}}\). D, Supernatants from hepatocytes isolated from gp130\(^{\text{flox}}\) and gp130\(^{-}\) mice unstimulated (control) and stimulated with IL-6 (200 ng/mL) for 24 hours were analyzed for SAA levels by ELISA (n=4). **\(P<0.01\) vs control, \#\(P<0.05\) vs gp130\(^{\text{flox}}\).
well well cell culture inserts (27 857 ± 2837 versus 50 142 ± 8325 cells; \( P < 0.05 \); Figure S3B) and BrdU incorporation (0.36 ± 0.01 versus 0.64 ± 0.07 OD_{450}; \( P < 0.05 \); Figure S3C). Accordingly, we observed a dose-dependent increase in SMC migration and proliferation using recombinant SAA, as assessed by transwell cell culture inserts (18 304 ± 3539 versus 4500 ± 957 cells; \( P < 0.01 \); Figure 6A), scratch assay (594 ± 146 versus 219 ± 84 \( \mu m^2 \); \( P < 0.01 \); Figure 6B), and BrdU incorporation (0.52 ± 0.11 versus 0.22 ± 0.07 OD_{450}; \( P < 0.01 \); Figure 6C).

To reconstitute plasma SAA levels of \( \approx 150 \mu g/mL \) observed in gp130\(^{\text{flox}} \) mice after carotid artery ligation (Figure 4A), we injected gp130\(^{-} \) mice with recombinant SAA1 after ligation. Compared with noninjected gp130\(^{-} \) mice, SAA injection restored neointima formation (15 106 ± 8327 versus 48 638 ± 4641 \( \mu m^2 \); \( P < 0.01 \); Figure 7A and 7B), as well as media thickening (39 554 ± 12 814 versus 94 726 ± 17 106 \( \mu m^2 \); \( P < 0.05 \); Figure 7A and 7C), for the most part as assessed by morphometry analysis at day 28 after ligation.
Maladaptive vascular remodeling is characterized by a hyperproliferative response of SMCs leading to thickening of the vascular wall mainly by neointima formation. This process depends on the presence of chronically altered mechanical forces occurring under pathophysiological conditions, such as arterial occlusive disease, atherosclerosis, and restenosis after angioplasty. Importantly, recent reports observed a strong correlation between vascular inflammation and neointima formation, pointing to an acute inflammatory process as a critical step in vascular remodeling.

The acute phase reaction is the immediate pool of systemic inflammatory reactions characterized by a very early synthesis and secretion of acute phase proteins, such as C-reactive protein and SAA from hepatocytes in the liver. In this regard, the activation of hepatocyte gp130 by the IL-6 cytokine family is crucially involved in this process. Interestingly, although acute phase proteins were shown to strongly correlate with cardiovascular events, little is known about their biological role in these processes. We have recently demonstrated a pivotal role for the gp130-dependent acute phase reaction in atherosclerosis. In particular, we observed an important influence of the predominant murine acute phase protein SAA on the migration and recruitment of mononuclear cells into the atherosclerotic lesion. In this consecutive study, we aimed to evaluate the contribution of the gp130-dependent acute phase reaction for vascular remodeling, which is mainly dominated by SMCs. Therefore, we subjected mice lacking hepatocyte gp130 on an apoE−/− background to an experimental model in which the blood flow in the left common carotid artery was discontinued by ligation near to the bifurcation (Figure S1). In this model, vascular remodeling is induced proximal to the site of ligation as a consequence of partial blood stasis, reduced shear stress, and enhanced wall tension. We observed a strong neointima formation and media thickening after carotid artery ligation in control mice, which was reduced by ~75% in hepatocyte-specific gp130 knockout mice, suggesting that the systemic gp-130−/− dependent acute phase reaction plays an important role in vascular remodeling.

Carotid artery ligation was accompanied by early enhanced SAA plasma levels in control mice, which was completely absent in gp130−/− mice. In clinical routine, it is well known that acute phase proteins are elevated after coronary angioplasty. In this study, we especially focused on SAA, because C-reactive protein seems to be of minor importance for the acute phase reaction in mice. SAA has been considered as a suitable marker for inflammation because it was significantly associated with chronic pathologies, such as Crohn disease, rheumatoid arthritis, and lupus erythematosus, and high levels of SAA correlated with the incidence of several cardiovascular diseases, such as atherosclerosis and myocardial infarction. However, increasing evidence has revealed a novel role for SAA to act on monocytes and leukocytes as a chemoattractant and to induce inflammatory cytokines. After carotid artery ligation, we observed enhanced plasma SAA levels, as well as enhanced local monocytes/macrophages infiltration, into the vessel wall in control mice. Strikingly, the lack of gp130-dependent acute phase reaction strongly inhibited the vascular recruitment of monocytes/macrophages. These results are consistent with our previous findings showing decreased plaque macrophages in hepato-

Discussion

In the present study, we demonstrate that vascular remodeling after carotid artery ligation critically involves the hepatocyte gp130-dependent systemic acute phase response. gp130-dependent release of the acute phase protein SAA from hepatocytes into the systemic circulation triggers vascular inflammation, as well as migration and proliferation, of SMCs and, subsequently, neointima formation and media thickening.

Maladaptive vascular remodeling is characterized by a hyperproliferative response of SMCs leading to thickening of
cyte-specific gp130-deficient mice after being fed a high-cholesterol diet. Thus, the activation of the gp130-dependent acute phase reaction with subsequent enhancement of SAA might be critical for vascular inflammatory processes.

Migration and proliferation of SMCs are critical steps in neointimal development after carotid artery ligation. Wang et al recently reported that adenosinergic overexpression of dominant-negative gp130 or STAT3 (signal transducer and activator of transcription) reduced SMC migration, and catheter-based delivery of these adenosinemas directly in the carotid artery subsequently reduced neointima formation after balloon injury, indicating the impact of vascular gp130/STAT3 signaling on vascular remodeling. However, this experimental design was targeted on local vascular gp130 effects without implying the gp130-dependent systemic acute phase reaction. Different from the study by Wang et al, we used here a model of hepatocyte-restricted gp130 deficiency with functional gp130 in the rest of the body, including the vasculature. Therefore, we were especially focusing here on the systemic acute phase reaction. We observed that enhanced SMC proliferation, neointima formation, and media thickening after carotid artery ligation in control mice were all impaired in hepatocyte-specific, gp130-deficient mice. Because early inflammatory infiltration is involved in the development of vascular remodeling, and activation of the acute phase reaction triggers the inflammatory response, we postulate here that acute phase proteins themselves are directly involved in vascular remodeling. Supporting this notion, we observed that SAA10 hepatocytes after gp130 stimulation with IL-6 released high SAA levels. These supernatant significantly triggered SMC migration and proliferation, which was completely inhibited by SAA immunoprecipitation or by the use of supernatants from IL-6 stimulated, gp130-deficient hepatocytes. At last, we observed that recombinant SAA alone was able to induce SMC migration and proliferation and SAA injection after carotid artery ligation restored vascular remodeling in gp130−/− mice. In this regard, it has been shown that SAA acts as a chemotractant for monocytes and leukocytes. With regard to SMCs, our results are consistent with a previous report by Kunom et al showing chemotactic properties of SAA for human aortic SMCs via the N-formyl peptide receptor-like protein (fPRL1). Thus, acute phase proteins, such as SAA, are not only markers of inflammation but also directly mediate migration and proliferation of SMCs after carotid artery ligation.

**Perspectives**

We demonstrate here that vascular remodeling after carotid artery ligation critically involves inflammation, as well as migration and proliferation, of SMCs mediated by the gp130-dependent induction of the systemic acute phase reaction. Acute phase proteins, such as SAA, are not only critically involved in early infiltration of inflammatory cells into the vessel wall but also trigger migration and proliferation of SMCs, leading to adaptive and compensatory vascular remodeling processes after carotid artery ligation. Therefore, modulation of the gp130-dependent acute phase reaction may represent a promising therapeutic target to prevent pathophysiological neointima formation.

**Acknowledgments**

We thank Silke Pretzer and Nathalie Stonka for excellent technical assistance.

**Sources of Funding**

This work was supported by the Deutschen Forschungsgemeinschaft DFG-grant Schie 386/7-2 (to K.G. and B.S.) and DFG-grant SFB566/B9 (to B.S.).

**Disclosures**

None.

**References**


Hepatocyte gp130 Deficiency Reduces Vascular Remodeling After Carotid Artery Ligation
Gustavo Salguero, Harald Schuett, Joanna Jagielska, René Schley, Ezequiel Tallone, Maren Luchtfeld, Helmut Drexler, Werner Müller, Karsten Grote and Bernhard Schieffer

Hypertension. 2009;54:1035-1042; originally published online October 5, 2009;
doi: 10.1161/HYPERTENSIONAHA.109.136002
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/54/5/1035

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/10/05/HYPERTENSIONAHA.109.136002.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Hepatocyte gp130-Deficiency Reduces Vascular Remodeling Following Carotid Artery Ligation

running title: hepatocyte gp130 and vascular remodeling

Gustavo Salguero1*, Harald Schuett1*, Joanna Jagielska1, René Schley1, Ezequiel Tallone1, Maren Luchtefeld1, Helmut Drexler1, Werner Müller2, Karsten Grote1** and Bernhard Schieffer1**

*equally contributing first authors

**equally contributing senior authors

From the Department of 1Cardiology and Angiology, Hannover Medical School, Germany and the 2Faculty of Life Sciences, Manchester University, United Kingdom

Corresponding author:
Bernhard Schieffer, M.D.
Department of Cardiology and Angiology
Hannover Medical School
Carl-Neuberg Strasse 1
30165, Hannover Germany
phone: +49-511-532-2129
fax: 49-511-532-5412
email: Schieffer.Bernhard@mh-hannover.de
Supplementary Methods

Reagents
Mouse recombinant interleukin (IL)-6 was obtained from CellSystems (St. Katharinen, Germany), recombinant human apo-serum amyloid A1 (SAA1) and platelet derived growth factor (PDGF)-BB were from PeproTech (London, UK), collagen was from Biochrom (Berlin, Germany) and Liberase Blendzyme 3 was from Roche (Mannheim, Germany). Following primary and secondary antibodies were used in this study: monoclonal mouse anti-α-SMC actin (Sigma, Taukirchen, Germany), polyclonal rabbit anti-Ki67 (Abcam, Cambridge, UK), polyclonal rat anti-MOMA-2 (Acris, Herford, Germany), polyclonal goat anti-SAA1 (R&D System, Minneapolis, MN), biotinylated goat anti-mouse (Vector Laboratories, Burlingame, CA), biotinylated rabbit anti-rat (Vector Laboratories, Burlingame, CA), biotinylated rabbit anti-goat (Invitrogen, Carlsbad, CA), Alexa Fluor® 555 conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA) and normal goat IgG (Santa Cruz, CA) as isotype control.

Morphometry and Immunostaining
The OCT-embedded common carotid arteries were cut systematically in serial 7 µm sections using a cryotome (Leica CM3050S, Leica Microsystems, Wetzlar, Germany). In all experiments, analyses were carried out in the ligated left common carotid artery of gp130−/− and gp130flox/flox mice, whereas the contralateral not ligated right carotid artery served as control (Figure S1A, please see http://hyper.ahajournals.org). We observed a robust and reproducible vascular remodeling in C57BL/6 wild type mice (WT) starting at a distance of 1000 µm from ligature and included both neointima formation and media thickening. Vascular remodeling reliably persisted up to 2500 µm from ligature and was investigated in four consecutive 500 µm segments of the ligated carotid (Figure S1B, please see http://hyper.ahajournals.org). Quantifying analyzes were made in 6-10 serial sections collected from segments 1000-2500 µm proximal to the site of ligation. For morphometric analysis, sections were stained with hematoxylin and eosin (H&E) and captured with a microscope (Leica DM4000B, Leica Microsystems, Wetzlar, Germany) at 100x magnification and analyzed using the image analysis software ImageJ (NIH, Bethesda, MD). Briefly, the perimeter of the lumen, the internal elastic lamina (IEL) and the external elastic lamina (EEL) were determined. The media area was calculated by subtracting the area defined by the IEL and the EEL. The intima area was calculated as the area defined by the luminal surface and the IEL.

For immunohistochemical analysis, sections were stained for smooth muscle cells (α-SMC actin) and monocytes/macrophages (MOMA-2). Proliferating cells were detected by immunofluorescent staining for the proliferation marker Ki67. Negative controls using IgG controls matching in species and concentration were run in parallel. Stained sections were captured with a microscope (Leica DM4000B, Leica Microsystems, Wetzlar, Germany) at 400x magnification and analyzed using the image analysis software ImageJ (NIH, Bethesda, MD). Data were expressed as percentage of positive stained area to total vascular area and as percentage of Ki67 positive nuclei to total vascular nuclei.

Cell Culture
Hepatocytes from gp130−/− and gp130flox/flox mice were isolated as described previously. Briefly, 3x10⁶ cells were cultured on collagen-coated 6 cm cell culture dishes (Nunc, Wiesbaden, Germany) in DMEM (Invitrogen, Carlsbad, CA) containing 4.5 g/L glucose supplemented with 10% FCS and penicillin/streptomycin for 24 hours. Cells were starved overnight and stimulated with 200 ng/mL IL-6 for 24 hours under serum-free conditions. Cell culture supernatants were collected and stored at -20°C for successive experiments.

Smooth muscle cells (SMCs) were isolated from the aorta of WT mice by an enzymatic dispersion method as described before. Cells were cultured on collagen-coated 25 cm² flasks (Nunc, Wiesbaden, Germany) in DMEM (Biochrom, Berlin, Germany) containing 1.0 g/L glucose supplemented with 20% FCS and penicillin/streptomycin and passage 3-10 were used for successive analysis.

Cell Migration Assay
Migration of SMCs was evaluated by transwell cell culture inserts and scratch assay. For transwell experiments, SMCs were starved for 24 hours and 1x10⁵ cells were placed in the upper chamber of transwell cell culture inserts (8-μm pore size; Corning, Corning, NY). The lower chamber contained
supernatants (1:2 dilution) from IL-6 stimulated and unstimulated hepatocytes isolated from gp130– and gp130flox mice, SAA (0.5 or 5 µg/mL) or PDGF-BB (50 ng/mL). Migration of SMCs was carried out for 48 hours at 37°C, 5% CO₂. Migrated cells into the lower chamber were quantified by counting in a Neubauer counting chamber. For scratch assay, SMCs were seeded in collagen-coated 1.5 cm cell culture dish with an integrated grid (Corning, Corning, NY), grown to complete confluence and subsequently starved for 24 hours. A vertical scratch on the cell-covered surface of the cell culture dish was made with the help of a trimmed blue tip. After removal of the scraped cells by washing, SMCs were stimulated with supernatants (1:2 dilution) from IL-6 stimulated and unstimulated hepatocytes isolated from gp130– and gp130flox mice, with SAA (0.5 or 5 µg/mL) or with PDGF-BB (50 ng/mL) for 48 hours at 37°C and 5% CO₂. Pictures were taken at the beginning and at the end of the experiments using an inverted cell culture microscope (CKX31, Olympus, Hamburg, Germany) at 40x magnification and a digital camera (C-5060, Olympus, Hamburg, Germany). The percentage of the recovered area indicating cell migration was determined using the image analysis software ImageJ (NIH, Bethesda, MD).

**Cell Proliferation Assay**

SMCs were seeded at 5x10³ cells/well on 96-well tissue culture plates (TPP, Trasadingen, Switzerland) and allowed to grow for 24 hours. After starvation for another 24 hours SMCs were stimulated with supernatants (1:2 dilution) from IL-6 stimulated and unstimulated hepatocytes isolated from gp130– and gp130flox mice, with SAA (0.5 or 5 µg/mL) or with PDGF-BB (50 ng/mL). Cell proliferation after 72 hours at 37°C and CO₂ was measured on the basis of DNA synthesis by 5-bromo-2’-deoxyuride (BrdU) incorporation with a commercial colorimetric quantification kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Each experimental condition was performed in triplicates. The amount of reaction product was determined by measuring the absorbance at 450 nm using a plate reader (µQuant, BIO-TEK, Bad Friedrichshall, Germany).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

SAA (SAA1) levels in the plasma and in supernatants of hepatocytes were determined with the help of a commercial ELISA (Tridelta, Bray, Ireland) according to the manufacturer instructions and a plate reader (µQuant, BIO-TEK, Bad Friedrichshall, Germany). For some experiments SAA plasma levels were determined 24 hours after LPS injection (2 mg/kg/bw in 200 µL PBS, i.p.).

**Immunoprecipitation**

Supernatants of hepatocytes were subjected to immunoprecipitation using antibodies against SAA (SAA1) and appropriate IgG as isotype control and protein G PLUS-Agarose (Santa Cruz, CA) following the manufacturer’s instructions.

**Statistical analysis**

Data are presented as mean±SEM of at least 3 independent experiments. Comparisons were made by the 2-tailed Student’s t-test for independent samples or 1-way ANOVA and post hoc Scheffé test as appropriate. Differences were considered statistically significant at a value of $P<0.05$.

**Supplementary References**


Figure S1. Overview of the carotid artery ligation model. A, Scheme of the aortic arch with all outlets. The site of ligation is indicated by a black bar. The dotted line identifies the segment of the left and right common carotid artery which was explanted and further analyzed. B, H&E stained sections of ligated left and not ligated right common carotid artery from WT mice of different distances from ligature 28 days following ligation. The site of ligation is indicated by a black bar.
Figure S2. No differences in SAA plasma levels following activation of the acute phase reaction by lipopolysaccharide (LPS) between gp130\textsuperscript{flox} and gp130\textsuperscript{−} mice. Plasma collected from gp130\textsuperscript{flox} and gp130\textsuperscript{−} mice 24 hours after injection of LPS (2 mg/kg/bw, i.p.) was analyzed for SAA levels by ELISA (n=5). **P<0.01 vs. control.
Figure S3. Migration and proliferation of smooth muscle cells are inhibited in response to supernatants from gp130^floox^ mice when SAA was depleted. Supernatants from hepatocytes isolated from gp130^floox^ mice stimulated with IL-6 (200 ng/mL) for 24 hours were subjected to immunoprecipitation (IP) with antibodies against SAA or control IgG. A, Supernatants were analyzed for SAA levels by ELISA (n=4). ^#P<0.05 vs. IgG B, Cell migration of SMCs after 48 hours was quantified using transwell cell culture inserts (n=4). ^#P<0.05 vs. IgG C, Cell proliferation of SMCs based on BrdU incorporation after 72 hours was quantified (n=4). ^#P<0.05 vs. IgG