Heme Oxygenase 1 Is Differentially Involved in Blood Flow–Dependent Arterial Remodeling
Role of Inflammation, Oxidative Stress, and Nitric Oxide

Mohamed Lamine Freidja, Bertrand Toutain, Antoine Caillon, Valérie Desquiret, Diane Lambert, Laurent Loufrani, Vincent Procaccio, Daniel Henrion

Abstract—Heme oxygenase 1 is induced by hemodynamic forces in vascular smooth muscle and endothelial cells. We investigated the involvement of heme oxygenase 1 in flow (shear stress)-dependent remodeling. Two or 14 days after ligation of mesenteric resistance arteries, vessels were isolated. In rats, at 14 days, diameter increased by 23% in high-flow arteries and decreased by 22% in low-flow arteries compared with normal flow vessels. Heme oxygenase activity inhibition using Tin-protoporphyrin abolished diameter enlargement in high-flow arteries and accentuated arterial narrowing in low-flow arteries (32% diameter decrease versus 22% in control). Two days after ligation, heme oxygenase 1 expression increased in high-flow and low-flow vessels, in association with a reduced mitochondrial aconitase activity (marker of oxidative stress) in high-flow arteries only. Inhibition of macrophage infiltration (clodronate) decreased heme oxygenase 1 induction in low-flow but not high-flow arteries. Similarly, inhibition of NADPH oxidase activity (apocynin) decreased heme oxygenase 1 induction in low-flow but not high-flow arteries. However, dihydroethidium staining was higher in high-flow and low-flow compared with normal flow arteries. In arteries cannulated in an arteriograph, heme oxygenase 1 mRNA increased in a flow-dependent manner and was abolished by Nω-nitro-L-arginine methyl ester, catalase, or mitochondrial electron transport chain inhibition. Furthermore, heme oxygenase 1 induction using cobalt-protoporphyrin restored altered high-flow remodeling in endothelial NO synthase knockout mice. Thus, in high-flow remodeling, heme oxygenase 1 induction depends on shear stress–generated NO and mitochondria-derived hydrogen peroxide. In low-flow remodeling, heme oxygenase 1 induction requires macrophage infiltration and is mediated by NADPH oxidase–derived superoxide. (Hypertension. 2011;58:225-231.) ● Online Data Supplement

Key Words: heme oxygenase 1 ■ blood flow ■ NO oxidative stress ■ mitochondria remodeling ■ resistance arteries

Heme oxygenase 1 (HO-1) is the inducible isoform of the rate-limiting enzyme involved in oxidative degradation of heme into equimolar amounts of carbon monoxide (CO), ferrous iron, and biliverdin. Biliverdin is subsequently reduced to bilirubin by the action of biliverdin reductase.1 HO-1, a 32-kDa protein, is also known as heat-shock protein 32.2 Its induction under various conditions of cellular stress, such as in the presence of reactive oxygen species (ROS) or reactive nitrogen species,3 occurs at the transcriptional level through the activation of NF-E2-related factor 2. This latter factor is translocated into the nucleus to form a heterodimer with one of the small Maf nuclear proteins, which in turn binds to Maf recognition element/stress responsive element localized in the promoter region of HO-1 and other target genes, thus activating their transcription.4,5 Many studies have highlighted the biological effects of heme degradation byproducts. Carbon monoxide is proposed to be, like NO, a vasorelaxant, but it may also regulate other vascular functions, such as platelet aggregation and smooth muscle proliferation.6 Bilirubin is a potent endogenous antioxidant.7 Furthermore, Fe released by HO-1 activity regulates several genes, including that of NO synthase (NOS).8

In both resistance arteries and large blood vessels, a chronic increase in blood flow enhances endothelial NOS (eNOS) expression and NO-dependent vasorelaxation. Furthermore, NO is essential for arterial outward hypertrophic remodeling after a chronic rise in flow.9,10 This remodeling allows the effect of altered hemodynamic forces on the vascular wall to be normalized.11 Conversely, blood flow reduction induces inward remodeling and reduces arteriolar contractility.9,12

Flow (shear stress) has been shown to increase HO-1 gene expression and CO production in cultured vascular smooth muscle13 and endothelial cells14 in a time- and a rate-
dependent manner. We, therefore, hypothesized that HO-1 may be induced in resistance arteries submitted to a chronic increase in blood flow in vivo. We also aimed to understand the role of HO-1 in flow (shear stress)-dependent remodeling in mesenteric resistance arteries (MRAs), taking into account the different signaling elements (eg, inflammation, \textsuperscript{15} NO, \textsuperscript{9} and oxidative stress\textsuperscript{16}) that have been reported previously to be implicated in this phenomenon.

**Methods**

For an expanded Methods section, see the online Data Supplement at http://hyper.ahajournals.org.

**Animals and Treatments**

Three-month–old male Wistar rats and eNOS knockout mice (January, Angers, France) were used. The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals (authorization No 00577), and the protocol was approved by the regional ethics committee.

**Protocol 1: Wistar Rats**

The rats were divided into groups treated or not with either Sn-Protoporphyrin (SnPP; 50 \text{ mmol/L} per kilogram, IP, daily; n=8) to inhibit HO-1 activity or apocynin (5 \text{ mmol/L} in drinking water; n=5) to inhibit NADPH oxidase activity. Control animals were treated with normal saline solution (0.9\% NaCl; n=12).

To investigate the role of macrophages in HO-1 induction, some animals were treated with liposome-encapsulated clodronate (2 \text{ mL/dL}; IP; n=6 per group) to suppress the peritoneal monocyte/macrophage population.\textsuperscript{15,17} Liposomes containing a physiological buffer solution instead of clodronate suspension were used as control. Liposome suspensions (0.69 \text{ mol/L} as clodronate) were prepared as described previously.\textsuperscript{18} Clodronate was provided by Roche Diagnostics GmbH (Mannheim, Germany).

**Protocol 2: eNOS Knockout Mice**

eNOS knockout mice were divided into 2 groups (n=5 per group), those treated or not treated with cobalt protoporphyrin (5 mg/kg, SC, twice a week) for HO-1 induction. Treatments were started 24 hours before surgery.

**In Vivo Mesenteric Artery Ligation**

Animals were anesthetized (isoflurane, 2.5\%) and pretreated with buprenorphine (Temgesic; 0.1 mg/kg, SC). A loop of intestine was exposed, and the local MRA blood flow was surgically modified according to Rotig et al.\textsuperscript{24} Citrate synthase activity was measured as described by Flamment et al.\textsuperscript{25}

For the aconitase activity measurement, 25 \text{ g} of homogenate proteins were added to the reaction buffer (Tris-HCl 50 \text{ mmol/L} [pH 7.4], manganese acetate 0.5 \text{ mmol/L}, sodium isocitrate 100 \text{ mmol/L}, and Triton 0.01\%, for 5 minutes at 37°C) in a final volume of 300 \text{ mL}. The rate of appearance of cis-aconitate was monitored at 240 nm during 5 minutes, using spectrophotometric rate determination according to Roitg et al.\textsuperscript{24} Citrate synthase activity was measured as described by Flamment et al.\textsuperscript{25}

**Statistical Analysis**

Results are expressed as mean±SEM. Means were compared using the Student t test or 1-way ANOVA, followed by the Tukey post-hoc test, as appropriate. For repeated measurements in pressure-diameter curves, the significance of the differences between groups was determined by 2-way ANOVA. Differences were considered statistically significant at P<0.05.

**Results**

Fourteen days after MRA ligation in control rats, the diameter increased in arteries submitted to a chronic increase in blood flow (high flow; HF), whereas the diameter was significantly reduced in arteries submitted to a chronic decrease in blood flow (low flow; LF), compared with control (normal flow; NF) arteries (Figure S1B). Two days after ligation, HO-1 gene and protein expressions were significantly higher in HF and LF vessels compared with NF vessels. After 14 days, HO-1 expression decreased significantly in HF and LF arteries compared with at 2 days. However, HO-1 gene expression in LF vessels was slightly but significantly higher than control level (Figure S2).

The HO-1 inhibitor SnPP increased HO-1 mRNA and protein level in NF, HF, and LF arteries compared with equivalent vessels in control rats (Figure S3). However, SnPP abolished the diameter enlargement in HF arteries and exacerbated diameter reduction in LF vessels (Figure 1A). On the other hand, the media thickness increased, albeit not significantly, in HF arteries and decreased significantly in LF arteries compared with NF arteries from control rats. SnPP did not affect these changes in media thickness (Figure 1B).
Effect of macrophage depletion using clodronate. Two days of macrophages in flow induction of HO-1, we tested the chronic changes in blood flow. Quantitative RT-PCR analysis of CD68 (a leukocyte marker) and HO-1 in mesenteric resistance arteries submitted to different flow levels in vitro. HO-1 decreased significantly only in LF but not in HF arteries under clodronate treatment (Figure 2B).

To investigate the involvement of ROS in HO-1 induction, the expression of NADPH oxidase subunits was evaluated in MRAs. Two days after ligation, NADPH oxidase subunit gene expression increased in HF and LF arteries compared with NF vessels. However, gene expression of Nox4 was significantly increased only in LF arteries. Fourteen days after local blood flow change, NADPH oxidase subunit expression levels in both HF and LF arteries were not different from that in NF vessels (Figure 3A through 3D). These data were completed using the NADPH oxidase inhibitor apocynin. Two days after blood flow alteration in apocynin-treated rats, HO-1 mRNA in LF arteries was similar to the level in NF vessels. However, apocynin did not affect HO-1 expression in HF arteries (Figure 3E).

To further evaluate the role of oxidative stress in flow-dependent remodeling, we localized ROS in the arterial wall. Two days after ligation, dihydroethidium staining (Figure 4A) and Rhodamine staining (Figure S6A), as well as 3-nitrotyrosine expression level (Figure S6B), were significantly higher in both HF and LF arteries compared with NF arteries. In addition, the ratio of mitochondrial aconitase:citrate synthase activity decreased significantly in HF (32% decrease compared with NF arteries) but not in LF arteries (Figure 4B). We then treated rats with apocynin and with the superoxide dismutase-mimetic Tempol (Figure S7). Both treatments prevented diameter enlargement in HF arteries, whereas they did not affect the reduction in diameter in LF vessels.

To further define the signaling pathway by which high flow (shear stress) upregulated HO-1 in MRAs, independent from macrophage activity in flow-dependent remodeling, MRAs were isolated and then mounted on an arteriograph and submitted to different flow levels in vitro. HO-1 (Figure 2A) and mitochondrial aconitase (Figure 2B) mRNA expression increased in HF and LF arteries compared with NF arteries. However, Nox4 expression was significantly higher in both HF and LF arteries compared with NF arteries (Figure 3A through 3D).

Interestingly, 2 days after ligation, the tissue inhibitor of metalloproteinase 1 (Figure S4A) and monocyte chemoattractant protein 1 (MCP-1; Figure S4B) gene expression levels were significantly higher in HF and LF than in NF vessels. This was much less pronounced 14 days after ligation. This confirms the role of matrix metalloproteinases and inflammation in flow-dependent remodeling.

We found macrophages in HF and LF arteries using immunolabeling and confocal microscopy (Figure S5) in agreement with a previous study. To assess the involvement of macrophages in flow induction of HO-1, we tested the effect of macrophage depletion using clodronate. Two days after arterial ligation in control rats, the expression level of CD68 (a leukocyte marker) was higher in HF and LF vessels compared with NF arteries. However, rats treated with liposome-encapsulated clodronate showed a significant decrease in CD68 mRNA in both HF and LF arteries (Figure 2A), suggesting decreased macrophage recruitment (as has been shown previously in similar conditions). The expression of HO-1 decreased significantly only in LF but not in HF arteries under clodronate treatment (Figure 2B).

Figure 2. Effect of macrophage depletion (using clodronate; CL) on heme oxygenase 1 (HO-1) gene expression in arteries submitted to chronic changes in blood flow. Quantitative RT-PCR analysis of CD68 (A) and HO-1 (B) in mesenteric resistance arteries submitted to a 2 day increase (high-flow; HF) or decrease (low-flow; LF) in blood flow compared with control arteries exposed to normal flow (NF). Arteries were isolated from rats treated or not (control; PBS) with CL. Values are mean±SEM (n=6 per group). *$P<0.05$: HO or LF arteries vs the corresponding NF arteries. #$P<0.05$: effect of CL vs PBS within HF arteries. §$P<0.05$: effect of CL vs PBS within LF arteries.

Figure 1. Effect of inhibition of heme oxygenase 1 (HO-1) activity on flow-dependent arterial remodeling. A, Percentage of diameter change in high-flow (HF) and low-flow (LF) arteries, from rats treated or not (control; CONT) with Sn-Protoporphyrin (SnPP) once a day for 2 weeks. $#P<0.05$: effect of SnPP vs CONT within HF arteries. $§P<0.05$: effect of SnPP vs CONT within LF arteries. B, Relative change of media thickness in HF and LF arteries vs normal flow (NF) vessels from rats treated or not treated with SnPP. $*P<0.05$: HF or LF arteries vs the corresponding NF arteries. Values are mean±SEM (n=8 per group).

To further define the signaling pathway by which high flow (shear stress) upregulated HO-1 in MRAs, independent from macrophage activity in flow-dependent remodeling, MRAs were isolated and then mounted on an arteriograph and submitted to different flow levels in vitro. HO-1 (Figure
3.4 compared with NF arteries, at pressure of /H11006 increase: 14.9 of HO-1 on HF remodeling in eNOS expression was increased (Figure S8B). Inhibitors except catalase, in the presence of which its
ingley, MCP-1 mRNA was not affected by any of these
higer than in zero-flow conditions (Figure 5B). Interest-
antly decreased flow-induced HO-1 mRNA, but it remained
expression. Furthermore, antimycin A and rotenone signifi-
cantly reduce flow-induced HO-1 mRNA expression in mesenteric resistance arteries submitted to a 2-day increase (HF) or decrease (LF) in blood flow compared with control arteries exposed to NF. Arteries were isolated from rats treated daily or not (control; CONT) with apocynin. *P<0.05, HF or LF arteries vs the corresponding NF arteries. #P<0.05: 14 days vs 2 days (A through D) or effect of apocynin (E) within HF arteries. §P<0.05: 14 days vs 2 days (A through D) or effect of apocynin (E) within LF arteries.

Figure 3. Role of NADPH oxidase activity on heme oxygenase 1 (HO-1) gene expression in arteries submitted to a chronic change in local blood flow. A through D, Quantitative RT-PCR analysis of p22phox, p47phox, gp91phox/Nox2, and Nox4 NADPH oxidase subunits in mesenteric resistance arteries submitted to a chronic increase (high-flow; HF) or decrease (low-flow; LF) in blood flow compared with control arteries exposed to normal flow (NF) for 2 or 14 days. Values are mean±SEM (n=6 per group). E, HO-1 gene expression in mesenteric resistance arteries submitted to a 2-day increase (HF) or decrease (LF) in blood flow compared with control arteries exposed to NF. Arteries were isolated from rats treated daily or not (control; CONT) with apocynin. *P<0.05, HF or LF arteries vs the corresponding NF arteries. #P<0.05: 14 days vs 2 days (A through D) or effect of apocynin (E) within HF arteries. §P<0.05: 14 days vs 2 days (A through D) or effect of apocynin (E) within LF arteries.

5A) and MCP-1 (Figure S8A) gene expression increased in a flow-dependent manner and stabilized at a flow rate of 100 µL/min. In agreement with in vivo findings, apocynin did not significantly reduce flow-induced HO-1 mRNA expression (Figure 5A). In another set of ex vivo experiments, arteries were submitted to an intraluminal flow of 100 µL/min, and HO-1 gene expression was measured. Aδ-Nitro-L-arginine methyl ester and catalase abolished flow-induced HO-1 gene expression. Furthermore, antmycin A and rotenone significantly decreased flow-induced HO-1 mRNA, but it remained higher than in zero-flow conditions (Figure 5B). Interestingly, MCP-1 mRNA was not affected by any of these inhibitors except catalase, in the presence of which its expression was increased (Figure S8B).

We then tested the effect of the pharmacological induction of HO-1 on HF remodeling in eNOS−/− mice. In untreated eNOS−/− mice, in agreement with our previous work,9 passive arterial diameter in HF arteries was equivalent to that of NF arteries 14 days after blood flow increase, whereas in eNOS−/− mice treated with the HO-1 inducer cobalt protoporphyrin, the arterial diameter of HF vessels was significantly higher than in NF vessels (percentage of diameter increase: 14.9±3.4 compared with NF arteries, at pressure of 75 mm Hg; Figure 6). This observation confirmed that NO is upstream in the signaling pathway regulating HO-1 expression by flow (shear stress).

Discussion
In this study, we showed that the HO system plays a critical role in high-flow–mediated remodeling of MRAs. Furthermore, byproducts of heme oxidative degradation might reduce low-flow–dependent remodeling by opposing arterial diameter narrowing.

Chronic increase and decrease in blood flow induce outward in inward arterial remodeling, respectively.19,26,27 These diameter changes aim to restore basal tensile and shear stress levels. It is important to note that, in this model, HF and LF arteries are compared with NF arteries obtained at the same time point from the same animal and subsequently submitted to comparable experimental and physiological conditions.

Our findings are in agreement with previous studies showing HO-1 overexpression in response to high-laminar shear stress in cultured cells.13,14 The implication of HO-1 in HF remodeling was confirmed by the inhibition of diameter enlargement when animals were treated with SnPP, a competitive inhibitor of HO activity (Figure 1A). The structural similarities between protoporphyrin compounds and heme result in activation of the HO-1 promoter, but SnPP was shown to efficiently inhibit HO activity.28 This suggests that the increase in diameter in HF arteries is most likely related to HO-1 activation and subsequently to the action of heme degradation byproducts.

The chronic increase in blood flow induces eNOS overexpression.9 Here, we found that an HF-induced diameter increase did not occur in eNOS−/− mice. However, HO-1 induction corrected impairment in HF remodeling despite the absence of the indispensable source of NO, eNOS.9,29 Thus, HO-1 is implicated in diameter enlargement downstream of eNOS.

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Our study is also in agreement with previous works showing that HF remodeling in MRAs requires the activation of NADPH oxidase and the production of ROS.$^{16,20,30}$ Indeed, both apocynin and Tempol prevented diameter enlargement (Figure S6). The concomitant presence of NO and superoxide anions promotes generation of peroxynitrite, ONOO$^-$, which activates matrix metalloproteinases in MRAs$^{16}$ and in large arteries.$^{31}$ Here, we showed that, in the early stages of HF remodeling, the tissue inhibitor of metalloproteinases 1 was upregulated in the endothelium$^{33}$ and attracts monocytes/macrophages, which are needed for flow-induced remodeling.$^{15}$ MCP-1 is a potent growth factor able to restore collagen conductance after femoral artery occlusion.$^{34}$ highlighting its role in arteriogenesis and remodeling after ischemia.

Because chronic increase in blood flow promotes ROS production in MRAs,$^{16}$ we assessed mitochondria stress by determining the ratio of aconitase:citrate synthase enzyme activity, a biomarker of oxidative stress.$^{35}$ In HF arteries, this ratio decreased, reflecting an increased ROS production by the mitochondria. On the other hand, despite an increased ROS level in LF arteries, the aconitase:citrate synthase ratio did not decline. These data suggest that production of

![A DHE staining](image)

**Figure 4.** Effect of flow changes on reactive oxygen species (ROS) production. A, ROS level measured using dihydroethidium staining (DHE) staining and confocal microscopy in mesenteric resistance arteries submitted to a chronic increase (high-flow; HF) or decrease (low-flow; LF) in blood flow compared with arteries exposed to normal flow (NF). Arteries were isolated 2 days after ligation. Positive (lipopolysaccharide; +LPS) and negative (−DHE) control experiments are shown in the top panel. Images shown are representative of 3 separate experiments. B, Ratio of aconitase:citrate synthase activity in mesenteric resistance arteries submitted to a chronic increase (HF) or decrease (LF) in blood flow compared with arteries exposed to NF. Values are mean±SEM (n=8 per group). *P<0.05, HF or LF arteries compared with the corresponding NF arteries.

![Aconitase/citrate synthase activity](image)

**Figure 5.** Effect of flow increase on heme oxygenase 1 (HO-1) gene expression. Quantitative RT-PCR analysis of HO-1 in mesenteric resistance arteries submitted ex vivo to an increase in flow for 1 hour, at 50 mm Hg, compared with control arteries in zero-flow conditions (A and B). Arteries were preincubated for 20 minutes in the presence or absence of the following inhibitors: apocynin (A), N$^\omega$-nitro-L-arginine methyl ester (L-NAME), catalase, antymycin A, or rotenone (B). Values are mean±SEM (n=5 per group). $^1$$^P$<0.05 vs zero flow. $^#$$^P$<0.05, effect of inhibitors vs arteries sheared at 100 μL/min.

![Diameter change (mesenteric arteries)](image)

**Figure 6.** Effect of cobalt protoporphyrin (CoPP) on flow-induced remodeling in endothelial NO synthase (eNOS)$^{-/-}$ mice. Pressure-diameter change (percentage) relationship determined in mesenteric arteries submitted to a chronic increase in blood flow (high-flow; HF). Arteries were isolated from untreated eNOS$^{-/-}$ mice (control; CONT) and CoPP-treated eNOS$^{-/-}$ mice submitted to arterial ligation for 14 days. Values are mean±SEM (n=4 to 5 per group). $^#$$^P$<0.05, effect of CoPP vs CONT, within HF group.
mitochondrial ROS is specific to HF and not to LF arteries. Indeed, shear stress increases mitochondria-derived hydrogen peroxide (H$_2$O$_2$) generation in endothelial cells. Mitochondria-derived H$_2$O$_2$ may induce HO-1 upregulation. Interestingly, HO-1 is involved in H$_2$O$_2$-induced vascular endothelial growth factor synthesis in rat vascular smooth muscle cells and in mouse aortic endothelial cells.

To further substantiate the above findings, we isolated rat MRAs and submitted them ex vivo to stepwise increases in intraluminal pulsatile flow. HO-1 gene expression increased in a rate-dependent manner until 100 µL/min. This induces maximum flow-mediated dilation ex vivo in MRAs in rats and mice. HO-1 induction was inhibited by the NOS inhibitor N$^o$-nitro-l-arginine methyl ester and by catalase, which catalyzes the transformation of H$_2$O$_2$ into H$_2$O. Furthermore, the mitochondrial electron transport chain inhibitors, antimycin A and rotenone, markedly reduced the increase in HO-1 expression. These data suggest that both H$_2$O$_2$, at least in part from mitochondrial origin, and NO play an important role in flow-induced HO-1 overexpression.

Flow-induced HO-1 increases CO production, which activates soluble guanylyl cyclase to produce cGMP. This latter reduces vascular tone, thus participating in diameter expansion during outward arterial remodeling in response to high blood flow.

Apocynin prevented diameter enlargement in HF remodeling (Figure S6A), whereas it did not affect HO-1 expression (Figure 3E). Indeed, HO-1 overexpression after the increase in blood flow had no significant effect on vascular tone because the wall extracellular matrix degradation was not possible under treatment with the NADPH oxidase activity inhibitor.

The LF arteries respond within a few hours after ligation, at least in part through the production of angiotensin II and the activation of its type 1 receptor. Meanwhile, MCP-1 expression in vascular cells was increased 2 days after ligation by a mechanism probably implicating the renin-angiotensin system. MCP-1 attracts macrophages, implicated at least in part through the production of angiotensin II and induces antioxidant genes in endothelial cells: role of reactive oxygen/nitrogen species. Free Radic Biol Med. 2007;42:260–269.


Belin de Chantemele EJ, Vessieres E, Dumont O, Guihot AL, Toutain B, Loufrani L, Henrion D. Reactive oxygen species are necessary for high situations by delaying arterial narrowing in arteries where blood flow is decreased and promoting the expansive response of collateral vessels, thus providing more efficient compensatory irrigation of downstream tissues. Both of these activities are required to prevent irreversible ischemia-induced injury. Although the expression of HO-1 is low in most tissues at physiological conditions, the use of drugs able to induce this enzyme, especially natural polyphenolic compounds, might prove to be a potential preventive and/or therapeutic tool against cardiovascular diseases.

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Disclosures
None.

References


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Heme oxygenase-1 is differentially involved in blood flow-dependent arterial remodeling: role of inflammation, oxidative stress and NO

Mohamed Lamine Freidja, Bertrand Toutain, Antoine Caillon, Valérie Desquiret, Diane Lambert, Laurent Loufrani, Vincent Procaccio, Daniel Henrion

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Expanded Methods:

**In Vivo Mesenteric Artery Ligation:**
From three adjacent first-order mesenteric arteries (MRAs), second-order branches of the first and third arteries were ligated with 7-0 surgical silk thread. This creates high flow (HF) in the middle vessel, with low flow (LF) in the other two vessels. Control (normal flow, NF) vessels were first-order MRAs obtained from the same animal (Fig. S1A, see online supplements). The physiological salt solution (PSS) was composed of NaCl (130 mM); NaHCO$_3$ (15 mM); KCl (3.7 mM); KH$_2$PO$_4$ (1.2 mM); MgSO$_4$ (1.2 mM); glucose (11 mM); and CaCl$_2$ (1.6 mM). The pH was 7.4; PO$_2$, 160 mmHg; and PCO$_2$, 37 mmHg. Each MRA was divided to several segments for functional and histomorphometric studies. They were also used for gene expression experiments.

**Arterial Diameter Measurement in Isolated Arteries:**
Mesenteric arteries were bathed in and perfused with a Ca2+-free PSS containing EGTA (2 mM) and sodium nitroprusside (10 µM). Diameter changes were measured when the intraluminal pressure was increased from 10 to 150 mmHg.

**Superoxide detection and confocal microscopy:**
Dihydroethidium (DHE) is cell permeable and is oxidized by superoxide anions to fluorescent products that are trapped by intercalation with DNA. MRAs sections were incubated with DHE (1 mM) in phosphate buffered solution (PBS) at 37°C for 30 minutes in a humidified chamber protected from light. Fluorescent images of ethidium bromide were obtained using a confocal microscope (Solamere Technology, UT, USA). Sections incubated with PBS alone served as negative controls and sections from animals treated with lipopolysaccharides (LPS) were used as positive controls.

**Western Blot analysis of 3-nitrotyrosine and HO-1:**
As previously shown (Cousin et al. Hypertension, 2010;55:109-15 and Belin de Chantemèlle EJ et al. Microcirculation, 2009;16(5):391-402) arterial segments were homogenized and proteins (20 µg total protein from each sample) were separated by SDS-PAGE. After migration, proteins were transferred to PVDF blotting membranes (Amersham). Membranes were then washed in TBST buffer and blocked for two hours at room temperature. Membranes were incubated for 90 minutes at room temperature with the primary antibody (anti-3-NT from Transduction Laboratories, 1/500 in TBST or anti-HO-1 from Stressgen, 1:1000), washed again (3 times for 10 min), and incubated with anti-mouse/rabbit peroxydase-conjugated anti-body (Amersham) in TBST. Membranes were washed (3 times for 10 min), and labeling was visualized using the ECL-Plus Chemiluminescence kit (Amersham).

**Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) analysis:**
200 ng or 100 ng of total RNA extracted from each sample were reverse transcribed into cDNA with QuantiTect® Reverse Transcription kit (Qiagen) according the manufacturer’s instructions. The Quantitative real-time PCR reactions and analysis were performed using the SYBR® Green JumpStart™ Taq ReadyMix™ kit (Sigma) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Primer sequences were designed using primer 3 software (Table S1).
The following primer sequences were used for qRT-PCR experiments:

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Table S1: Nucleotide sequences of Primers used for qRT-PCR

**HO-1 and MCP-1 gene expression in isolated mesenteric arteries (ex vivo):**
Previous studies \(^1,2\) as well as our preliminary experiments (data not shown) have shown that gene activation of HO-1 and MCP-1 has a time frame of minutes, reaching a peak of expression in 1 to 2 hours after the blood flow has been changed.

**Aconitase and Citrate Synthase activity measurements in MRAs:**
Ten to twenty milligrams of MRAs were homogenized in 100 to 200 μl of extraction buffer (mannitol 220 mM, saccharose 75mM, Tris 10 mM, EGTA1mM, pH 7.2) and centrifugated (650g, 20 minutes, 4°C). The total protein content in the supernatant was determined using bicinchoninic acid, with bovine serum albumin used as the standard (BC Assay kit, Montluçon, France).
References

Figure S1: Schematic representation of the mesenteric circulation in rats and pressure-diameter relationship after arterial ligation:

A: The location of the ligations of second-order mesenteric artery branches is indicated by arrows. The artery located between two ligated vessels (low flow, LF) was considered to be a high flow (HF) artery. Equivalent non-ligated arteries located at a distance from the ligatures had normal flow (NF).

B: Pressure-diameter (passive arterial diameter) relationship was determined in mesenteric arteries isolated from untreated rats submitted to arterial ligation for 14 days (A). Values are mean ± SEM (n = 7 per group).

*P<0.05, HF or LF arteries compared with the corresponding NF arteries.
Figure S2: HO-1 gene expression (A) and protein expression level in flow-dependent arterial remodeling:
qRT-PCR (A) and Western-blot (B) analysis of HO-1 in mesenteric resistance arteries submitted to a chronic increase (HF) or decrease in blood flow (LF) compared to control arteries exposed to normal flow (NF), for 2 or 14 days. Values are mean ± SEM (n = 11 per group). Typical blot for HO-1 and beta actin are shown below panel B.
*P < 0.05, HF or LF arteries compared with the corresponding NF arteries.
# P < 0.05: 14 days versus 2 days, within HF arteries
§ P < 0.05: 14 days versus 2 days, within LF arteries
Figure S3: Effect of Sn-protoporphyrin treatment on HO-1 gene (A) and protein (B) expression: qRT-PCR analysis (A) and Western-blot analysis (B) of HO-1 in mesenteric resistance arteries submitted to a chronic decrease (LF) or increase in blood flow (HF) compared to control arteries exposed to normal flow (NF). Arteries were isolated from rats treated or not (control, CONT) with SnPP once a day for 2 weeks.

$ P < 0.05$: SnPP versus CONT, within NF arteries
$ § P < 0.05$: SnPP versus CONT, within LF arteries
Figure S4: MCP-1 and TIMP-1 gene expression in flow-dependent arterial remodelling:

qRT-PCR analysis of TIMP-1 (A), and MCP-1 (B) in mesenteric resistance arteries submitted to a chronic increase (HF) or decrease in blood flow (LF) compared to control arteries exposed to normal flow (NF), for 2 or 14 days. Values are mean ± SEM (n = 11 per group).

*P<0.05, HF or LF arteries compared with the corresponding NF arteries.

# P < 0.05: effect of chronic change in blood flow (14-days, compared to 2-days increase in blood flow in HF arteries)
Macrophages location using immunostaining and confocal microscopy in mesenteric resistance arteries submitted to a chronic increase (HF) or decrease in blood flow (LF) compared to control arteries exposed to normal flow (NF), for 2 days. 2 different markers of macrophages were used: CD11b (FITC labeling for the antibodies) and F4/80 (Texas-red labeling for the antibodies). Nuclei were labeled with DAPI (blue labeling). Merging is shown in the lower panels. Cartoons give the position of the whole artery during analysis. Images are representative of 5 different experiments.

References:
S6A: Reactive oxygen species (ROS) visualized using rhodamine staining (green) in mesenteric resistance arteries submitted to a chronic decrease (LF) or an increase in blood flow (HF) compared to control arteries exposed to normal flow (NF). Nuclei were stained with DAPI (blue staining). ROS level was quantified by image analysis In negative control experiments rhodamine was omitted. A positive control was obtained with arteries isolated from LPS-treated rats. Fluorescence quantification is shown in the bargraph. Mean±SEM is presented (n=8 per group).
*P<0.05, HF versus NF

References:
S6B: 3-nitrotyrosine (3-NT) expression level determined using Western-blot analysis in the 3 types of mesenteric arteries. 3-NT level was quantified and expressed as a ratio to β-actin. Mean±SEM is presented (n = 8 to 10 per group).

*P<0.05, HF or LF arteries compared to the corresponding NF arteries.

References:
Figure S7: Pressure-diameter relationship after treatment with apocynin or tempol:

Pressure-diameter (passive arterial diameter) relationship was determined in mesenteric arteries isolated from rats submitted to arterial ligation for 14 days. Rats were treated with the NADPH-oxidase inhibitor apocynin (A) or with the superoxide dismutase analog Tempol (B). Values are mean ± SEM (n = 8 per group).

*P<0.05, HF or LF arteries compared with the corresponding NF arteries.
Figure S8: Effect of flow increase on MCP-1 gene expression:

qRT-PCR analysis of MCP-1 in mesenteric resistance arteries submitted ex vivo to an increase in PSS-flow, for 1 hour, at 50 mmHg (A and B). Arteries were preincubated for 20 minutes in the presence or not of these inhibitors: L-NAME, Catalase, Antimycin A or Rotenone (B). Values are mean ± SEM (n = 5 per group).

*P<0.05, sheared arteries, compared to control arteries in zero-flow conditions.

# P < 0.05: effect of inhibitors, compared to arteries sheared at 100 µl/min.