Cardiac output generates blood flow in large compliance arteries followed by small resistance muscular arteries able to adapt their diameter to the metabolic need of the downstream located tissues. Resistance arteries are subjected to mechanical forces, pressure, and flow (shear stress), inducing respectively myogenic tone and flow-dependent dilation. Flow stimulates the endothelium to produce contractile (PGH2, TXA2, endothelin, reactive oxygen species) and relaxing factors including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI2). Long-term changes in blood flow induce arterial wall remodeling to normalize shear stress. In large conductance arteries, remodeling is associated with neointimal hyperplasia and depends on NO production and matrix metalloprotease (MMP) activation. In arterioles or resistance arteries, flow-dependent remodeling is involved in physiological processes, such as blood vessel growth during development, exercise training, or pregnancy and in pathological situations including hypertension, diabetes, ischemic diseases, or tumor growth.

In resistance arteries blood flow reduction induces inward remodeling and reduced contractile capacity whereas chronic increases in blood flow triggers outward hypertrophic remodeling. The mechanisms involved in flow-induced remodeling have been mainly investigated in large elastic arteries and in cultured endothelial cells which may not be relevant for arterioles in vivo. Studies in small arteries show the central role of shear stress, circumferential wall stress, transient de-differentiation, and turnover of smooth muscle cells and growth factors. The role of NO is essential in large arteries as shown in arterio-venous fistula in the rabbit or the rat or in ligated carotid arteries in mice lacking eNOS. NO takes part differently in remodeling along the vascular tree. In resistance arteries, the role of NO remains controversial. Although eNOS expression increases in resistance mesenteric arteries submitted to high flow, chronic NO-synthesis inhibition with L-NAME does not prevent remodeling. Nevertheless, in this latter study chronic L-NAME induces a large increase in blood pressure preventing the use of a fully blocking dose of L-NAME. In addition, high blood pressure attributable to L-NAME induces remodeling by its own, thus interfering with flow-induced remodeling. Thus we aimed to further investigate the role of the NO-pathway in flow-induced remodeling of resistance arteries using higher doses of L-NAME and mice lacking the gene encoding for eNOS.
In addition, NO has a key role in the control of matrix metalloprotease (MMP) activity involved in flow-induced remodeling,3,25 in large elastic arteries. Thus the absence of effect of NO in microvascular remodeling would also exclude MMPs from the process in resistance arteries. Nevertheless, no study has yet been conducted in resistance arteries undergoing remodeling to test the role of MMPs. Thus, we assessed the hypothesis that MMPs stimulated by NO could play a role in flow-induced microvascular remodeling. We conducted a functional and a biochemical study in resistance arteries chronically submitted to low or high blood flow, for 4 and 14 days, to determine the role of NO and MMPs in remodeling. These two time points have been chosen to study early and late mechanisms of flow-remodeling. Indeed Buus et al23 showed that the remodeling induced by a decrease in blood flow occurred after only 2 days, whereas the remodeling induced by an increase in blood flow was a slower process requiring approximately 2 weeks to be completed. Changes in flow were performed by alternative ligations of mesenteric arteries in mice or rats.11,21 NO blockade was obtained with high dose of L-NAME (LN) in drinking water in parallel with an antihypertensive treatment, perindopril (P), which was a slower process requiring approximately 2 weeks to be completed. Changes in flow were performed by alternative ligations of mesenteric arteries in mice or rats.11,21 NO blockade was obtained with high dose of L-NAME (LN) in drinking water in parallel with an antihypertensive treatment, perindopril (P), preventing deadly rises in blood pressure.23 Mice lacking the gene encoding for eNOS were used as well. Doxycycline (DOX) was used to prevent MMP activity. We found that both eNOS and MMP activation are essential for resistance arteries remodeling induced by a chronic increase in blood flow, whereas low flow-induced remodeling was prevented by perindopril.

Materials and Methods

In Vivo Ligation of Rat Mesenteric Arteries

Adult male rats Wistar were anesthetized (sodium pentobarbital, 50 mg/kg IP) and submitted to surgery to modify blood flow in the mesenteric arteries, as previously described.11,21 Arteries were submitted to high (HF), low (LF), or normal flow (NF) (supplemental Figure IA and IB) and divided in 4 groups and treated in drinking water as described below:

1. DOX (n=15) : doxycycline (40 mg/kg/d);
2. LN + P (n=15): L-NAME (60 mg/kg per day) plus perindopril (6 mg/kg/d);
3. P (n=15): perindopril (6 mg/kg/d);
4. CT (n=15): control without treatment

Treatments were started 24 hours before surgery.

After 14 days, rats were euthanized and mesenteric arteries collected for functional (n=8 per group) and histomorphometric analyses (n=4 per group) studies. In a separate series of experiments, at 4 and 14 days, biochemical (n=7 per group) and immunohistochemical (n=6 untreated rats only) studies were performed.

In addition, mice lacking the gene encoding for eNOS (−/−) (n=5) and their littermate controls (+/+) (n=4) were submitted to arterial ligation during 14 days as described above.

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 nb 00577).

Arterial Diameter Measurement in Isolated Arteries

Segments of mesenteric arteries were cannulated at both ends in a video-monitored perfusion system as previously described.21 Briefly, arteries were bathed and perfused with a Ca2+-free physiological salt solution (PSS) containing EGTA (2 mmol/L) and sodium nitroprusside (10 µmol/L). Diameter changes were measured when intraluminal pressure was increased from 10 to 150 mm Hg.

Western Blot Analysis of eNOS

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibody (Transduction Laboratories, 1:2000). Proteins were visualized using the ECL-Plus Chemiluminescence kit (Amersham).21

Immunohistochemistry and In Situ Zymography

Arterial segments were embedded vertically in Tissue-tek (Sakura). MMP-2 and MMP-9 were detected with primary goat polyclonal antibodies (Santa Cruz Biotechnology, 1:100) followed by the fluorescent secondary antibody (1:200). In control experiments sections were incubated with an inhibitor (EDTA) and an activator (APMA) of metalloproteinases. Immunostaining and in situ gelatinolysis were revealed by the appearance of fluorescence visualized and quantified in the media using confocal microscopy.26

Arteries Histomorphometric Analysis

Transversal sections of arteries fixed under a pressure of 75 mm Hg were stained with orcein solution. Then internal and external medial circumferences were measured to determine media surface.11

Statistical Analysis

Results were expressed as means±SEM. Significance of the differences between groups was determined by analysis of variance (ANOVA for consecutive measurements for pressure-diameter curves) or 1-way ANOVA followed by Bonferroni or paired t test. Probability values less than 0.05 were considered to be significant.

Results

Mean arterial blood pressure was not significantly affected by treatments with L-NAME plus perindopril (113±10.5 mm Hg at Day 4 and 102±9 mm Hg at Day 14) or doxycycline (103±9.4 mm Hg at Day 4 and 107±7.4 mm Hg at Day 14), compared with control (101±3 mm Hg at Day 4 and 109±2.6 mm Hg at Day 14). In rats treated with perindopril mean arterial pressure was not affected after 4 days (82±10 mm Hg) and was decreased after 14 days (74±4.8 mm Hg).

Untreated Rats

Structural Properties of Arteries and Arterial Passive Diameter

Passive arterial diameter was measured in control rats’ arteries 14 days after ligations. High, low, and normal flow arteries were designated as HF, LF, and NF arteries, respectively. In isolated mesenteric resistance arteries stepwise increases in pressure induced diameter (passive diameter) enlargement, when measured in a calcium-free PSS + EGTA (2 mmol/L).

In control rats, passive arterial diameter was higher in HF arteries than in NF arteries, whereas in LF arteries it was significantly decreased (Figure 1A).

Expression of eNOS

Four and 14 days after ligation, the expression of endothelial NO synthase (eNOS or NOS III) was increased in HF arteries.
In rats treated with doxycycline, no significant remodeling was observed in HF arteries (Figure 3B and 3E), whereas perindopril alone did not affect remodeling in HF arteries (Figure 3D and 3E). In LF arteries remodeling was not affected by the treatment associating L-NAME and perindopril (Figure 3C and 3F), but it was inhibited by perindopril alone (Figure 3D and 3F). Arterial diameter in NF arteries was not affected by doxycycline as compared with untreated rats (Figures 1A and 3B).

In rats treated with L-NAME and perindopril no significant remodeling was observed in HF arteries (Figure 3C and 3E), whereas perindopril alone did not affect remodeling in HF arteries (Figure 3D and 3E). In LF arteries remodeling was not affected by the treatment associating L-NAME and perindopril (Figure 3C and 3F), but it was inhibited by perindopril alone (Figure 3D and 3F). Arterial diameter in NF arteries was not affected by perindopril associated or not with L-NAME (Figures 1A, 3C, and 3D).

In eNOS/−/− mice, passive arterial diameter was higher in HF arteries than in NF arteries, whereas in LF arteries it was decreased. In eNOS/−/− mice, passive arterial diameter in HF arteries was equivalent to that in NF arteries, whereas in LF arteries it was decreased (Figure 4A).

**Expression of eNOS**

In HF and LF arteries of L-NAME and perindopril-treated rats, the expression of eNOS was not different from that in NF arteries, except in LF arteries after 14 days of ligation where it was decreased (Figure 4B and 4C).

Four and 14 days after ligation, in HF and LF arteries of perindopril-treated rats, the expression of eNOS was respectively increased and decreased compared with that in NF arteries.

Four and 14 days after ligation, in HF arteries of doxycycline-treated rats, the expression of eNOS was significantly increased compared with that in NF arteries. In LF arteries, it was equivalent to that in NF arteries.

Four and 14 days after ligation, in NF and LF arteries, the expression of eNOS was not significantly affected by any treatment. In HF arteries of L-NAME plus perindopril-treated rats and doxycycline-treated rats, the expression of eNOS was significantly lower than in HF control arteries.

**Regulation of MMP-2 and MMP-9 Activity**

In situ zymography showed lytic areas corresponding to an activity of both MMP2 and MMP9. In NF arteries of control rats, a significant gelatinolytic activity was detected at Day 4 and Day 14. This MMPs activity was assigned a value of 100% (Figure 5).

In HF arteries of control rats and perindopril-treated rats, the gelatinolytic activity was significantly increased at Day 4 and was equivalent to that in NF at Day 14. In LF arteries, the gelatinolytic activity was not significantly different from NF at Day 4 and Day 14.

Four and 14 days after ligation, in L-NAME and perindopril-treated rats and doxycycline-treated rats, the gelatinolytic activity in HF and LF arteries was equivalent to that in NF.
Four days after ligation, in NF and LF arteries, the gelatinolytic activity was not significantly affected by any treatment, whereas in HF arteries, the gelatinolytic activity was prevented to increase by a treatment with L-NAME and perindopril or with doxycycline.

**Discussion**

This study shows a relationship between NO production, eNOS expression, MMP activity, and the remodeling of resistance arteries induced by a chronic increase in blood flow in vivo. On the other hand, inward remodeling induced by a chronic decrease in blood flow was associated with a decreased eNOS expression. This latter remodeling was prevented by angiotensin I converting enzyme (ACE) inhibition.

Microvascular structural and functional changes induced by chronic changes in blood flow have a key role in pathological processes such as hypertension, ischemic diseases, diabetes, or tumor growth. Thus a better understanding of the mechanisms involved in flow-induced remodeling of resistance arteries is especially important.

We used a model previously described in rats and mice, allowing the comparison of resistance arteries submitted to different blood flow levels in the same conditions and in the same animal. In these resistance arteries, chronic increases and decreases in blood flow induce outward and inward arterial remodeling, respectively. These diameter changes allow normalization of wall shear stress and are accompanied by a compensatory change in medial mass, which restores circumferential wall stress. In this model blood flow increases by approximately 60% and arterial remodeling fully normalizes shear stress. This is different from flow-induced remodeling in large arteries, obtained with arterio-venous fistulae inducing a 6- to 10-fold increase in blood flow and an incomplete shear stress normalization. Besides tissue specificity this difference in the amplitude of the stimulus may account for differences in the mechanism(s) involved in the process, especially concerning the remodeling induced by blood flow reductions (see below).

As the NO pathway and MMPs are involved in the remodeling of large arteries in response to a chronic increase in blood flow, we measured changes in expression levels of eNOS.
First we demonstrated the key role of eNOS activation in high flow-induced remodeling of resistance arteries. High flow-induced remodeling was prevented by NO synthesis inhibition with L-NAME and by the absence of the gene encoding for eNOS. To fully block NO production we used a high dose of L-NAME in association with an ACE inhibitor, thus preventing the mortality attributable to L-NAME. In addition, perindopril prevented the rise in blood pressure induced by L-NAME, which is important as high blood pressure, per se, induces remodeling, thus rendering difficult a conclusion on flow-induced remodeling. Indeed, high blood pressure attributable to L-NAME induces inward eutrophic remodeling. The present study is in agreement with previous findings obtained in large elastic arteries. On the other hand our result contrasts with a previous report stating that, in resistance arteries, chronic L-NAME does not affect high flow-induced remodeling, using the same model. Nevertheless, the dose of L-NAME used was lower than in the present study and the rise in blood pressure attributable to L-NAME was interfering with flow-induced remodeling as stated above. In addition, treatment with L-NAME was started after ligations, allowing HF-remodeling to start before the inhibition of NO pathway had reached an effective level.
Another main finding of the present study is that eNOS activation and the consecutive activation of MMP9 are essential for HF-remodeling. We found that MMP inhibition with doxycycline prevented high flow-induced remodeling of resistance arteries. In addition, HF-remodeling was also associated with a rise in MMP2/9 activity and MMP9 expression 4 days after ligation. This was followed by a return to baseline activity and expression at Day 14. These data suggest a more noteworthy involvement of MMP9 than MMP2 in high flow-induced remodeling of resistance arteries, although the activity measurement does not allow discerning between MMP2 and MMP9. After an increase in blood flow, MMPs could be activated by NO, or directly by shear stress. For the purpose of determining the sequence of events involved in flow-remodeling, we assessed the expression of eNOS and MMPs activity in L-NAME-treated and doxycycline-treated rats. In HF arteries, L-NAME prevented eNOS overexpression (Figure 4B and 4C) and MMPs activation (Figure 5B) whereas doxycycline inhibited only the rise in MMPs activity without preventing eNOS expression to rise in the HF arteries (Figure 5B). These data suggest that a sequential activation of eNOS and then of MMPs occurred in HF remodeling. These findings are supported by a recent study showing that eNOS mediated MMPs activation. Without preventing eNOS overexpression in HF arteries, a downward shift in eNOS expression was nevertheless observed in the arteries isolated from doxycycline-treated rats. This observation is in agreement with previous studies establishing the inhibiting effects of doxycycline on NOS expression.

Thus, the chronic increase in blood flow in resistance arteries stimulated eNOS expression and activated NO production that in turn enhanced MMP9 activity leading to cell proliferation, media hypertrophy, and diameter enhancement. In resistance arteries, a chronic decrease in blood flow induces an important diameter reduction. This remodeling has been found hypotrophic with a reduced smooth muscle cell number and size associated with apoptosis. In the present study we did not find a significant hypotrophy, although a tendency exists in the 4 groups of rats. Nevertheless, the present study and our previous work agree with the other findings associated with the LF-remodeling such as the hyporeactivity and the decreased protein expression (mainly eNOS). We found that this remodeling was not affected by NO synthesis or MMP activity inhibition with L-NAME or doxycycline, respectively. These findings contrast with the remodeling induced by blood flow...
reduction in large arteries\textsuperscript{32} where intimal hyperplasia develops in association with MMP2/9 activation.\textsuperscript{33} Angiotensin I converting enzyme inhibition prevented low flow-induced remodeling. This finding supports the hypothesis that inward remodeling in resistance arteries results from an unbalanced vasoconstrictor tone attributable to the reduction in flow stimulating the endothelium followed by transglutaminase-dependent stabilization of the remodeling.\textsuperscript{34,35} This prevention of the remodeling induced by blood flow reduction can also be caused by the blood pressure reduction observed in perindopril-treated rats (significant at day 14; 35 mm Hg blood pressure reduction compared with control). This latter hypothesis is compatible with the one given above as suppressing a vasoconstrictor influence and reducing blood pressure should increase arterial diameter. Indeed Perindopril alone reduces blood pressure and exerts a vasodilator influence thus inhibiting LF-remodeling. When perindopril is associated with L-NAME its vasodilator effect is counteracted by the vasoconstrictor influence of L-NAME, resulting in a normal blood pressure and the restoration of LF-remodeling. Our data showing a downregulation of the NO pathway are in agreement with the previous studies showing that shear stress is a main determinant of eNOS expression.\textsuperscript{36}

In addition, ACE inhibition, which did not affect the diameter enlargement induced by the chronic increase in blood flow, prevented the rise in media surface. This is especially important as arterial wall hypertrophy is correlated to the outcome of

\textbf{Figure 5.} Gelatinase activity in NF, HF, and LF arteries 4 and 14 days after ligations, using confocal microscopy. Positive and negative controls are represented (A). Quantification of gelatinolytic activity was performed using image density analysis in untreated rats and rats treated with L-NAME and perindopril, perindopril alone, or doxycycline (6 rats per group). Data are presented as percentage of NF arteries 4 (B) and 14 days (C) after ligations (mean±SEM). \textsuperscript{*}P<0.05, HF or LF vs NF. \textsuperscript{#}P<0.05 for HF, LF, or NF in treated groups vs the corresponding artery in the control group.
cardiovascular events. Nevertheless, in the present study, perindopril was used to prevent the rise in blood pressure attributable to L-NAME and the dose required, when used alone, induced a significant decrease in blood pressure as compared to control. Thus further investigations are required to better understand the role of the renin-angiotensin system in flow-dependent hypertrophic remodeling in resistance arteries.

In conclusion, these findings show that in resistance arteries chronic increases in blood flow, in vivo, induce a structural and functional remodeling requiring NO production and MMPs activation. On the other hand, low flow-induced remodeling was independent of NO and MMPs.

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Key Role of the NO-Pathway and Matrix Metalloprotease-9 in High Blood Flow-Induced Remodeling of Rat Resistance Arteries
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Figure I:

Diagram of the mesenteric circulation in rats (A). The locations of the ligations of the second-order mesenteric artery branches are indicated by arrows. Ligated arteries were designated as low flow (LF) arteries. The artery located between two ligated vessels was designated as a high flow (HF) artery. Other arteries had a normal flow (NF).

Typical examples of histological cross sections of arteries exposed for 14 days to NF, HF or LF in control rats vessels (B).