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# Type 2 diabetes severely impairs structural and functional adaptation of rat resistance arteries to chronic changes in blood flow

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Short title : Diabetes impairs flow-mediated remodeling

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## Abstract

Endothelial dysfunction in resistance arteries leads to end organ damages in type 2 diabetes. The capacity of the microcirculation to adapt or remodel in response to changes in blood flow or shear stress is essential for an optimal organ perfusion. Chronic increases in blood flow enhance arterial diameter and endothelium-dependent dilation. Since type 2 diabetes impairs endothelial sensitivity to flow (shear stress) and increases oxidative stress, we hypothesized that Zucker diabetic fatty rats (ZDF) would present an impaired flow-induced remodeling in resistance artery. Aim: The goal of the study was to compare the structural and functional adaptations of mesenteric arteries from lean (LZ) and ZDF rats, to chronic changes in blood flow. Methods: Two mesenteric resistance arteries were ligated in order to increase blood flow in a third artery. This artery was thus submitted, *in vivo*, to high flow (HF) and compared to normal flow (NF) arteries located at distance. After 3 weeks arteries were studied *in vitro* (n= 10 rats per group). Results: Arterial diameter (468 vs 394±8µm) and endothelial (acetylcholine)-dependent dilation (91±8 vs 75±6% dilation) were higher in HF than in NF arteries in LZ rats. In ZDF rats, arterial diameter (396±9 vs 440±17µm) and acetylcholine-mediated dilation (42±8 vs 75±7% dilation) were lower in HF than in NF arteries. Nevertheless, endothelial NO-synthase and NADP(H)-oxidase subunits (gp91, p67) expression level and superoxide production were higher in HF than in NF arteries in both LZ and ZDF rats HF suggesting an efficient flow-sensing process in both ZDF and LZ rats. But, in ZDF rats basal oxidative stress was higher compared to LZ rats: a) dihydroethidium staining was higher in both NF and HF arteries in ZDF than in LZ rats and b) acetylcholine-mediated dilation was improved by an acute antioxidant (tempol) in NF and HF arteries from ZDF rats (no effect in LZ rats NF arteries). As a consequence, oxidative stress, slightly increased in LZ rats HF arteries was strongly increased in ZDF rats HF vessels. This led to an excessive superoxide production impairing NO-dependent dilation and HF-remodeling. Finally, a chronic treatment with tempol restored HF arteries diameter (426±13 in NF vs 471±16µm in HF arteries) and endothelium-dependent dilation in ZDF rats. Conclusion: In type 2 diabetic rats increasing blood flow chronically failed to induce outward remodeling and to improve endothelium-dependent dilation; mainly because of superoxide overproduction.

## Introduction

Type 2 diabetes is the most frequently encountered metabolic disorder, currently affecting 5% to 10% of the population <sup>1</sup>. Associated with obesity, type 2 diabetes is characterized by an insulin resistance inducing several metabolic changes among which hyperinsulinemia, hyperglycaemia, dyslipidaemia and hypertension, all leading to an increased risk of cardiovascular events <sup>2</sup>. The morbidity and mortality associated with type 2 diabetes are essentially related to the vascular lesions that develop over time in this condition <sup>3</sup>. The microcirculation is mainly involved, and as a consequence, vital organs are damaged. While the consequences of type 2 diabetes on large elastic arteries <sup>4, 5</sup> have been extensively studied, less is known about its effects on the microcirculation. In addition, no study has yet investigated the effects of type 2 diabetes on the ability of resistance arteries to adapt their structure and function in response to a chronic rise in blood flow.

The primary function of the microcirculation is to optimize nutrient and oxygen supply within tissues in response to the metabolic demand. For this purpose, resistance arteries are able to adapt their diameter in response to mechanical stimuli such as pressure and flow (shear stress). Mechanisms involved are respectively myogenic tone in response to pressure and flow-mediated dilation (FMD) <sup>6</sup>. Furthermore, resistance arteries are able to adapt to chronic increases in blood flow, leading to diameter enlargement (outward remodeling) and higher endothelium (NO)-dependent relaxation <sup>7-10</sup>. This remodeling is involved in response to an increase in the metabolic demand of different tissues, in either pathological conditions such as diabetes or in growth, following exercise training or during pregnancy. The production of NO by the endothelium and the activation of matrix metalloproteinase (MMPs) are required for flow-mediated remodeling, as previously shown in small resistance <sup>9</sup> and large elastic arteries <sup>11</sup>. In conditions involving a reduced endothelial ability to produce vasodilator agents, such as aging, increasing chronically local blood flow has been shown to improve endothelium (NO)-dependent dilation. This was associated with reduced ROS production and improved eNOS expression level and function <sup>12</sup>.

Type 2 diabetes is associated with an increased ROS production <sup>13</sup> that might impair the ability of resistance arteries to adapt their structure and function in response to chronic increases in blood flow because of a decreased NO-bioavailability <sup>14</sup>. Nevertheless, as ROS, besides NO, are also required for flow-mediated remodeling <sup>15</sup>, the effect of ROS overproduction on remodeling cannot be deduced from previous studies. Indeed, our previous study performed in obese Zucker rats has shown that flow-induced remodeling (diameter enlargement) occurred despite a large overweight and a slight

hypertension and hyperglycemia. We are thus hypothesizing that endothelial alteration and oxidative stress might compromise resistance arteries remodeling in of type 2 diabetic rats.

To verify this hypothesis we used a model of ligation of the mesenteric bed <sup>16-18</sup> allowing the comparison of resistance arteries chronically submitted to high or normal blood flow levels, in the same physiological conditions. We used Zucker Diabetic Fatty (ZDF) rats treated or not with the antioxidant tempol, hypothesizing that coupling the chronic rise in blood flow to a reduction in ROS level will improve the ability of resistance artery to respond to chronic changes in blood flow.

## **Methods:**

### **Animals**

Twenty adult male Zucker fatty diabetic (ZDF) and 20 lean Zucker (LZ) rats, 12-14 week-old, were purchased from Charles River (L'Arbresles, France). Rats were anesthetized (Isoflurane, 2.5%) and submitted to surgery in order to modify blood flow as previously described <sup>9, 17, 19</sup>. Briefly, three consecutive first order arteries were used. Ligatures (7-0 silk surgical thread) were applied to second order branches of the first and third arteries, as shown on Figure 1A. The artery located between two ligated arteries was designed as high flow (HF) artery. Other arteries located at distance of the ligated arteries were used as control (normal flow, NF) arteries. Half of the rats were simultaneously treated with 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol, 10 mg/kg per day).

Before sacrifice, glycemia was quantified on a sample of arterial blood with a glucometer <sup>10</sup>.

Twenty-one days after surgery, animals were anesthetized (Isoflurane, 2.5%). The right femoral artery was catheterized for blood pressure measurement <sup>20</sup>. Animals were then sacrificed by CO<sub>2</sub> inhalation, the gut excised and mesenteric arteries gently dissected. From each rat, HF and NF arteries were isolated and divided in several segments used respectively for pressure-diameter relationship measurement, pharmacology, biochemistry as well as for immuno-histological analyses.

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 (Ministère de l'Agriculture, France, authorization No. 6422) and with the Principles of laboratory animal care (NIH publication no. 85-23, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>).

### **Pressure-diameter relationship and flow-dependent dilation in NF and HF arteries**

A first segment of NF and HF arteries was cannulated at both ends and mounted on a video monitored perfusion system<sup>21</sup>. Briefly, arterial segments were bathed in a 5 mL organ bath containing a physiological salt solution (PSS) and superfused at a rate of 3 mL/min. Arterial diameter was measured and recorded continuously using a video monitoring system (Living System Instrumentation Inc., Burlington, VT). Before each experiment, the integrity of the endothelium was assessed by testing the relaxing effect of acetylcholine (ACh, 1  $\mu$ mol/L) after a phenylephrine (PE, 1  $\mu$ mol/L)-induced precontraction.

Arteries were then submitted to 75 mmHg of pressure and further contracted with PE. Intraluminal flow was subsequently increased by step (3 to 100  $\mu$ L/min) in order to induce flow (shear stress)-mediated dilation (FMD). After 30 minutes of recovery FMD was repeated in the presence of N(omega)-nitro-L-arginine methyl ester (L-NAME, 100  $\mu$ mol/L, 20 min). Finally, arteries were perfused and superfused with a Ca<sup>2+</sup>-free PSS containing ethylenbis-(oxyethyleninitrolo) tetra-acetic acid (EGTA, 2 mmol/L) and sodium nitroprusside (10  $\mu$ mol/L). Arteries were submitted to a stepwise increase in pressure (10 to 150 mmHg) in order to determine the passive diameter of the vessel, i.e., in the absence of smooth muscle tone. Pressure and diameter measurements were recorded using a Biopac data acquisition system (Biopac MP 100, La Jolla, CA, USA) and analyzed (Acqknowledge® software, Biopac). Flow-induced dilation was expressed as percent dilation of the precontraction<sup>22</sup>.

### **Pharmacological profile of isolated NF and HF arteries**

A second segment of HF or NF mesenteric arteries (2 mm long) was dissected and mounted on a wire-myograph (DMT, Aarhus, DK)<sup>23</sup>. Briefly, 2 tungsten wires (25  $\mu$ m diameter) were inserted into the lumen of the arteries and fixed to a force transducer and a micrometer, respectively. Arteries were bathed in a physiological salt solution. Wall tension was applied as described previously<sup>24</sup>. The artery's viability was tested using a potassium rich solution (KCl, 80 mmol/L). A cumulative concentration-response curve (CRC) to ACh (0.001 to 10  $\mu$ mol/L) was then performed. Thirty minutes after washout a second CRC to ACh was repeated in the presence of N(omega)-nitro-L-arginine methyl ester (L-NAME, 100  $\mu$ mol/L, 20 min), indomethacin (10  $\mu$ mol/L) or tempol (10  $\mu$ mol/L).

### **Histology**

At the end of the functional analysis on the arteriograph, the artery was bathed in  $\text{Ca}^{2+}$ -free PSS containing 10  $\mu\text{mol/L}$  sodium nitroprusside (SNP). Pressure was set at 75 mmHg, and the artery was fixed in a 4% buffered formaldehyde solution as previously described<sup>10</sup>. Sections (7  $\mu\text{m}$  thick) were stained with orcein. External diameter, lumen diameter and media thickness were determined after images acquisition (Olympus T100 microscope, Sony camera) and analyzed using the Histolab software (Microvision, Paris, France) for cross sectional area calculation as previously described<sup>25</sup>.

### **Western Blot analysis**

The remaining NF arteries of each mesenteric vascular bed were pooled and then homogenized. Proteins (25  $\mu\text{g}$  total protein from each sample) were separated by SDS-PAGE using a 4% stacking gel followed by a 10% running gel. Proteins were detected with specific antibodies (Transduction Laboratories, eNOS 1:1000, Cav-1 1:4000, p67 and gp91 and actin 1:1000 in T-TBS-BSA 5%). Protein expression was visualized using the ECL-Plus Chemiluminescence kit (Amersham)<sup>26</sup>.

### **Detection of reactive oxygen species (ROS) using confocal microscopy**

Other NF and HF arterial segments were embedded vertically in Tissue-tek (Sakura) and frozen in isopentane. ROS detection was performed on transverse cross sections 7  $\mu\text{m}$  thick incubated with dihydroethyidine (DHE) as previously described<sup>10</sup>. Briefly, DHE, in the presence of superoxide, is oxidized to fluorescent ethidium bromide. Ethidium bromide is trapped by intercalation with DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. Positive staining was visualized using confocal microscopy and QED-image software (Solamere Technology, Salt Lake City, UT). Image analysis was performed using Histolab (Microvision, France). Pixels quantification was executed after separating the media and the endothelial layer<sup>10, 25</sup>.

### **Statistical analysis**

Results are expressed as means  $\pm$  SEM. Significance of the difference between arteries was determined by ANOVA (1-factor ANOVA or ANOVA for consecutive measurements, when appropriate). Means were compared by paired t-test or by the Bonferroni test for multigroup comparisons. Values of  $p < 0.05$  were considered to be significant.

## **Results:**

### **Physiological parameters**

In order to determine the metabolic status of the LZ and ZDF rats, we measured their body weight, glycemia and blood pressure. Rat body weight was significantly higher in ZDF than in LZ rats ( $441\pm 17$  versus  $325\pm 11$ g,  $p<0.05$ ) and was not significantly affected by the chronic treatment with tempol in both ZDF ( $428\pm 20$  versus  $441\pm 17$ g) and LZ rats ( $312\pm 14$  versus  $325\pm 11$ g).

Similarly, blood glucose was significantly enhanced in ZDF compared to LZ rats ( $302\pm 23$ mg/dL versus  $112\pm 13$ mg/dL). Blood glucose was not modified by the chronic treatment with tempol in both LZ ( $117\pm 10$ mg/dL) and ZDF rats ( $298\pm 20$ mg/dL).

Type 2 diabetes significantly increased mean blood pressure ( $92\pm 2$  mmHg in LZ versus  $111\pm 2$  mmHg in ZDF,  $p<0.05$ ). However, mean blood pressure was not significantly affected by the chronic treatment with tempol, in both the LZ ( $94\pm 3$  mmHg) and ZDF ( $113\pm 2$  mmHg) rats.

### **Arterial diameter and structure**

Passive arterial diameter in HF and NF arteries was determined in order to assess the arterial remodeling induced by the chronic increase in blood flow. In LZ rats HF arteries diameter was significantly higher than in NF arteries (fig. 1B). By contrast, in ZDF rats HF arteries diameter did not increase compared to NF arteries. In addition, it was significantly lower than in NF arteries for an intraluminal pressure of 75 mmHg ( $P<0.05$ ). Thus arterial outward remodeling did not occur in ZDF rats. The chronic rise in blood flow significantly increased the media cross-sectional area of HF arteries from the LZ rats only. Nevertheless, type 2 diabetes induced an increase in the media cross sectional area of the NF artery. Furthermore, no difference in media cross-sectional area was observed between NF and HF arteries from ZDF rats. (figure 1C).

### **Endothelium-dependent relaxation**

The vasodilator function of the endothelium was assessed by measuring the response of NF and HF arteries to stepwise increases in flow and to increasing concentrations of ACh. In LZ rats, flow-mediated dilation (Figure 2A) and ACh-induced relaxation (Figure 2B) were significantly higher in HF than in NF arteries. By contrast, in ZDF rats increasing blood flow chronically reduced endothelium-dependent dilation as evidenced by a decreased in flow-mediated dilation (Figure 2A) and ACh-induced relaxation in HF compared to NF arteries (Figure 2B). As a control, endothelium-independent



dilation in response to sodium-nitroprusside was performed in the same arteries. This dilation was not affected by the chronic rise in blood flow (no difference between NF and HF arteries) or by diabetes (no significant change between LZ and ZDF rats) (figure 2C). On the other hand, the contraction induced by phenylephrine was higher in ZDF than in LZ rats and higher in HF than NF arteries (figure 2D).

The precontraction of NF and HF arteries before inducing dilation with flow, ACh or sodium nitroprusside was not significantly different among groups (figure 2E).

### **Effect of acute endothelial NO synthase and cyclooxygenase blockade on endothelium-dependent dilation**

To determine the role played by eNOS and NO in endothelium-dependent dilation, concentration-response curves to ACh were performed in the presence of the eNOS inhibitor L-NAME. In LZ rats, L-NAME significantly suppressed ACh-induced relaxation in HF arteries whereas it reduced, but not suppressed the ACh-mediated relaxation, in NF arteries (figure 3A). In ZDF rats, L-NAME significantly suppressed ACh-induced relaxation in NF arteries but not in HF arteries. In HF arteries, ACh-induced dilation was reduced by half for the 3 highest concentrations, whereas it was not significantly affected with the lowest concentrations (figure 3B). After addition of indomethacin no significant dilation could be detected in response to ACh in LZ rats NF arteries whereas the dilation was improved by indomethacin in ZDF rats NF arteries. Indomethacin had no further effect in HF arteries isolated from either LZ or ZDF rats (figure 3 A and B).

### **Caveolin-1 and eNOS protein expression levels:**

As previously shown by our group<sup>9,10</sup>, eNOS and caveolin-1 expression levels increased in HF arteries compared to NF vessels (figure 3C). There was no significant difference in expression levels between LZ and ZDF rats suggesting that a change in eNOS or caveolin-1 expression level cannot explain the changes in NO-dependent dilation.

### **Effect of acute ROS reduction on endothelium-dependent dilation:**

In order to test the effect of ROS on NO-dependent dilation, we measured the acute effect of the antioxidant tempol on ACh-induced relaxation (figure 4 A & B). In LZ rats the treatment with tempol

did not affect ACh-induced dilation in NF or HF arteries (figure 4A). On the other hand, in ZDF rats, tempol increased significantly ACh-induced relaxation in both NF and HF arteries (figure 4 B).

#### **NADP(H) oxidase (gp91 and p67) protein expression levels:**

We have previously shown that the NADP(H)-oxidase subunits gp91 and p67 expression levels increase in HF arteries <sup>10</sup>. The expression levels of these 2 proteins increased significantly in HF arteries compared to NF vessels in both LZ and ZDF rats (figure 4C). In addition, there was a significant increase in gp91 and p67 expression levels in ZDF rats compared to LZ rats in both NF and HF arteries (figure 4C).

#### **Effect of a chronic treatment with tempol on flow-induced remodeling:**

In order to test the effect of oxidative stress on the vascular response to a chronic increase in blood flow we submitted ZDF rats to a chronic treatment with the antioxidant tempol. In tempol-treated ZDF rats, HF arteries diameter was significantly higher than HF arteries diameter in non-treated rats (figure 5A). No effect of the chronic treatment with tempol was observed on the diameter of the NF arteries, in the ZDF rats (figure 5A). Cross-sectional area was not significantly affected by tempol (supplement, panel A).

#### **Effect of a chronic treatment with tempol on ACh-induced dilation:**

The chronic treatment with tempol restored endothelial function. Indeed, in tempol-treated ZDF rats ACh-induced dilation was similar in HF and NF arteries. In addition, L-NAME totally suppressed ACh-induced dilation in both HF and NF arteries (figure 5B). In LZ rats treated with tempol ACh-induced dilation was higher in HF than in NF arteries and totally suppressed by L-NAME (supplement data, panel E). Flow-mediated dilation in tempol-treated rats followed the same pattern (supplement, panel F) and eNOS expression level was not significantly affected by the treatment (supplement, panel B).

#### **Effect of a chronic treatment with tempol on ROS production:**

ROS detection using DHE-staining showed a positive ROS level in the NF and HF arteries of the ZDF rats. This staining was significantly higher in HF than in NF arteries. In tempol-treated ZDF rats no positive nucleus could be detected, similarly to negative control arteries (DHE omitted). On the

other hand, in a positive control artery (isolated from a LPS-treated rat) the majority of the nuclei were fluorescent (figure 5C). Chronic tempol increased gp91 and p67 expression level (supplement, C and D).

## **Discussion**

In the present study we found that type 2 diabetes impaired the ability of mesenteric arteries to remodel and to improve NO-dependent dilation in response to a chronic increase in blood flow. Indeed, ROS production, high in Zucker diabetic rats (ZDF), was further increased in high-flow arteries (HF) thus inducing an additional reduction of the endothelium-dependent relaxation instead of enhancing the endothelial function as observed in LZ rats. Nevertheless, a chronic antioxidant treatment restored the ability of mesenteric arteries from ZDF rats to increase their diameter and endothelium-dependent dilation in response to a chronic rise in blood flow.

In physiological conditions, a chronic rise in blood flow, in resistance arteries, enhances vascular diameter and improves endothelium-dependent dilation<sup>10, 17, 22</sup>. This remodeling is essential to adjust organ perfusion during physiological processes such as development<sup>27</sup>, pregnancy<sup>28</sup> or exercise training<sup>29</sup> as well as during pathological processes, mainly ischemic diseases. A similar remodeling also occurs in response to vasodilator treatments<sup>30, 31</sup>. Indeed, this remodeling is also called arteriogenesis<sup>32</sup> and the model used in the present study has the advantage to involve resistance arteries and to allow the study of the effects of blood flow, on the arterial wall, independently of pressure or metabolic changes and without ischemia.

In type 2 diabetes, the endothelium is less capable of inducing vasodilatation, especially in resistance arteries, which control end-organs supply in blood flow<sup>33</sup>. In addition, the outward hypertrophic remodeling observed in arteries from type 2 diabetic animals<sup>34-37</sup> might cause the increased contractility of the smooth muscle and the higher myogenic response observed in patients suffering type 2 diabetes<sup>34, 38</sup>. Our findings are in agreement with these previous observations. In ZDF rats mesenteric arteries (NF and HF arteries) we found that hypertrophy (high cross-sectional area) was associated with a higher phenylephrine-mediated constriction.

In order to improve endothelium-dependent dilation, and consequently local blood flow, vasodilator treatments, therapies improving insulin sensitivity or exercise are commonly used. These treatments are associated with a higher eNOS expression, which is, at least in part, the consequence of a chronic rise in blood flow<sup>9, 10, 22</sup>. This latter has also been shown to increase eNOS expression level and NO-dependent dilation in ageing, a situation associated with reduced endothelium responsiveness<sup>12</sup>. This latter study has reported that a chronic rise in flow, using the model described in the present study, improves endothelium (NO)-dependent dilation in 8 month-old rats<sup>12</sup>. Nevertheless, no study has yet investigated the effect of a chronic rise in blood flow in resistance arteries in type 2 diabetes. It is reasonable to speculate that this should also increase arterial diameter and/or endothelium-dependent tone as reported in healthy young animals<sup>39</sup> and in old rats<sup>12</sup>.

The first main result of the present study is that endothelium-dependent dilation was not improved but further reduced after submitting ZDF rat's arteries to a chronic rise in blood flow. Indeed, by contrast with lean rats, HF arteries from ZDF rats exhibited a reduced endothelium-dependent dilation compared to NF arteries, showing that a further endothelial dysfunction occurred. The defect observed in ZDF rats was not due to a change in smooth muscle response to NO as the relaxation induced by the NO-donor sodium nitroprusside was not affected. Furthermore eNOS expression was higher in HF than in NF arteries in both ZDF and LZ rats. Nevertheless, despite an increased eNOS expression in HF arteries in ZDF rats, the involvement of NO in the dilation was severely reduced as evidenced by the absence of effect of L-NAME on acetylcholine-induced dilation. On the other hand, in LZ rats L-NAME strongly reduced acetylcholine-induced dilation and this effect was higher in HF than in NF arteries, in agreement with previous studies<sup>10, 12</sup>. The expression level of eNOS and caveolin-1, modulated by high flow as previously shown<sup>9</sup>, was increased in HF arteries from ZDF rats. This suggests that the initial response of the arteries to the rise in flow or shear stress was maintained. The defect inducing a reduced dilation is thus located downstream eNOS. The cyclooxygenase inhibitor indomethacin suppressed the remaining dilation after L-NAME in LZ rats NF arteries, in agreement with previous observations in the same vessel<sup>40</sup>. In ZDF rats NF arteries vasoconstrictor prostanoids are most probably produced in response to ACh, in agreement with previous studies showing that vasoconstrictor cyclooxygenase derivatives reduce endothelium-mediated dilation in diabetes<sup>41</sup>. In HF arteries, isolated from both LZ and ZDF rats, indomethacin had no effect as L-NAME suppressed

totally the dilation. This might be related to the increased eNOS expression found in HF arteries that may act on COX expression and/or activity although this issues remains controversial <sup>42</sup> and requires further investigation.

In order to find the cause of the paradoxically low endothelium (NO)-dependent dilation found in ZDF rats HF arteries, we investigated the effect of reducing ROS level, on the dilation, using tempol, which catalyzes the transformation of ROS into H<sub>2</sub>O<sub>2</sub> <sup>26</sup>. Tempol, acutely applied to isolated arteries, increased ACh-induced dilation in NF and HF arteries of ZDF rats. Tempol did not affected ACh-dependent relaxation in LZ rats. Nevertheless a chronic treatment with tempol improved both ACh- and flow-mediated dilation in HF arteries. Thus the reduction in NO-dependent dilation observed in ZDF rats arteries was the consequence of an excessive ROS production counteracting NO bioavailability. In the HF artery, the reduction in NO-dependent dilation was the result of the initial scavenging of NO by ROS, as seen in NF arteries, plus the additional increase in ROS level due to the chronic rise in blood flow.

The cause of this excessive ROS production may be multiple. In type 2 diabetes ROS production is abnormally high and interacts with NO-dependent dilation in resistance arteries <sup>43</sup>. In HF artery a further increase in ROS level was observed. We found that NADP(H)-oxidase subunits expression (gp91 and p67, figure 4) and DHE staining (figure 5) were higher in HF than in NF in ZDF rats. This difference between NF and HF vessels is in agreement with our previous studies analyzing NF and HF arteries isolated from other rat strains <sup>10, 26</sup>. Our data is also in agreement with a study analyzing carotid arteries submitted to a rise in blood flow through arterio-venous fistulae <sup>15</sup>. Thus, in ZDF rats high basal ROS level was associated with high NADP(H) oxidase expression level, which was further increased by the chronic rise in flow. As a consequence, the rise in eNOS expression and the associated higher NO production in HF arteries was counteracted by this excessively high ROS level.

The hyperphagia and overweight, which characterize ZDF rats, in addition of diabetes, can also induce endothelium dysfunction. In a previous study, we have shown that rats with a similar overweight but without major hyperglycemia (obese Zucker rats) present an impairment of their endothelial function, similar to that observed in ZDF rats <sup>10</sup>. Nevertheless, the effects of the chronic increase in blood on the endothelium dependent dilation were lower on the arteries from obese Zucker rats than on the HF artery of the ZDF rats. Thus, obesity per se might explain part of the dysfunction

found in HF arteries in ZDF rats whereas diabetes is certainly the cause of the most important lost of dilation.

In order to confirm that excessive ROS production in ZDF rats prevented endothelium-dependent dilation to be improved despite high eNOS expression level, ZDF rats were chronically treated with tempol. After a chronic tempol infusion, acetylcholine-mediated dilation was increased to control level (NF arteries in LZ rats) and completely blocked by L-NAME, showing that NO-induced dilation was fully restored (figure 5B). Thus the combination of a chronic rise in blood flow, increasing eNOS expression, with an antioxidant treatment allowed improving endothelium-dependent dilation in ZDF rats.

Another key finding of the present study is that the chronic rise in blood flow in ZDF rats did not induce the expected increase in arterial diameter. A possible explanation is that type 2 diabetes has already induced an outward hypertrophic remodeling<sup>34-37</sup> and that a further rise in diameter is not possible. Nevertheless, after a chronic treatment with tempol a further increase in diameter was observed, although the rise was not equivalent to that found in LZ rats (present study) or in other rats strains<sup>9, 10</sup>. The flow-sensing process was probably not affected in ZDF rats as the response to the chronic rise in flow was “normal” with a rise in eNOS and caveolin-1 expression equivalent to that observed in LZ rats. This observation rules out, at least in part, a possible reduction in flow sensing by advanced glycation end products (AGEs), which have been shown to reduce the activity of several processes in type 2 diabetes. AGEs have been reported to alter the matrix proteins collagen, vitronectin, and laminin, through AGE-AGE intermolecular covalent bonds, or cross-linking<sup>37, 44</sup>. Furthermore AGE cross-linking on type I collagen and elastin causes an increase in the area of extracellular matrix, resulting in increased stiffness of the vasculature<sup>44</sup>. Finally, the absence of an increase in diameter in HF arteries cannot be the consequence of the overweight observed in ZDF rats as in a previous study performed in obese but not diabetic Zucker rats we have shown that outward remodeling occurred normally<sup>10</sup>.

In the present study, ROS reduction restored in part flow-induced outward remodeling. Indeed, the excessive ROS production found in HF arteries may affect the remodeling process per se. Indeed, if the association of NO plus ROS is important for the remodeling an excessive ROS level might reduce NO availability to a level low enough to prevent ONOO<sup>-</sup> production and MMPs activation, both

essential for flow-induced remodeling<sup>9, 15, 26</sup>. This is supported by our observation showing that L-NAME was totally unable not block acetylcholine-dependent dilation in ZDF rats HF arteries (figure 3B).

The consequences of the present study are multiple. Vasodilator treatments in type 2 diabetes not only have to reduce hypertension but they are also expected to improve local blood flow supply in order to prevent end organ damages. This latter effect might be more efficient with treatments possessing antioxidant properties or if they are associated with antioxidant. Similarly, exercise is recommended to patients suffering type 2 diabetes<sup>45</sup>, based on the observation that exercise improves local blood flow as previously shown in gracilis muscle resistance arteries<sup>46</sup>. Our finding suggests that this recommendation would benefit an association with a reduced oxidative stress. Indeed, our finding provides a rationale for the epidemiological observations showing that associating exercise with healthy diet has a better chance to improve NO availability and reduces oxidative stress<sup>47</sup>. We have previously shown that a reasonable quantity of vegetal polyphenols with antioxidant properties had a beneficial effect of post-ischemic revascularization of the rat hindlimb after femoral ligation. This revascularization involves arteriogenesis<sup>48</sup>, which is equivalent to HF-remodeling.

In Summary, increased reactive oxygen species production induced by type 2 diabetes and by the rise in shear stress seriously altered the ability of resistance arteries to adapt their structure and function in response to a chronic increase in blood flow. This impairment was reversed by an antioxidant treatment suggesting that a vasodilator treatment should have antioxidant properties in order to be fully efficient in diabetic patients.

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**Conflict of Interest:** none declared

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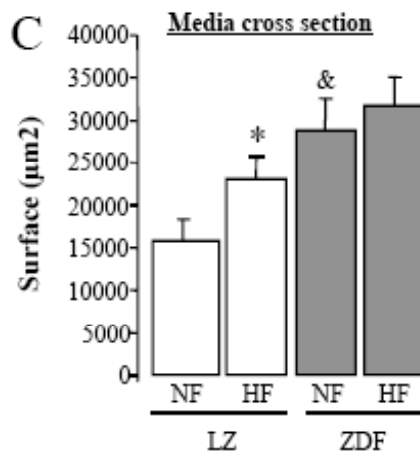
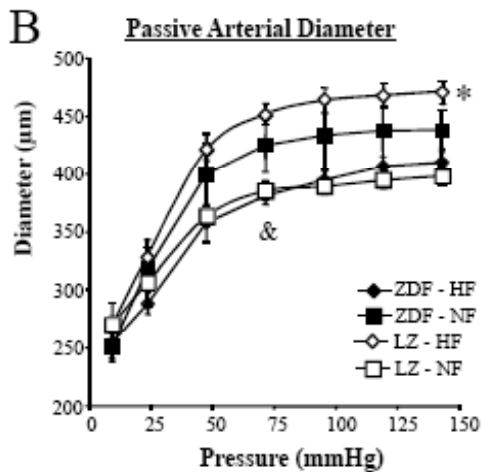
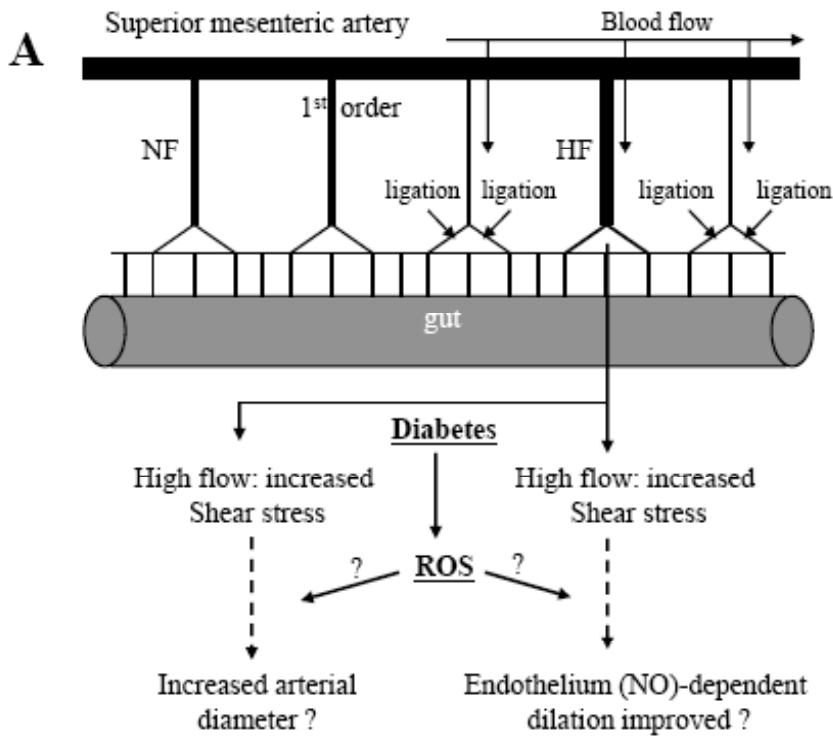


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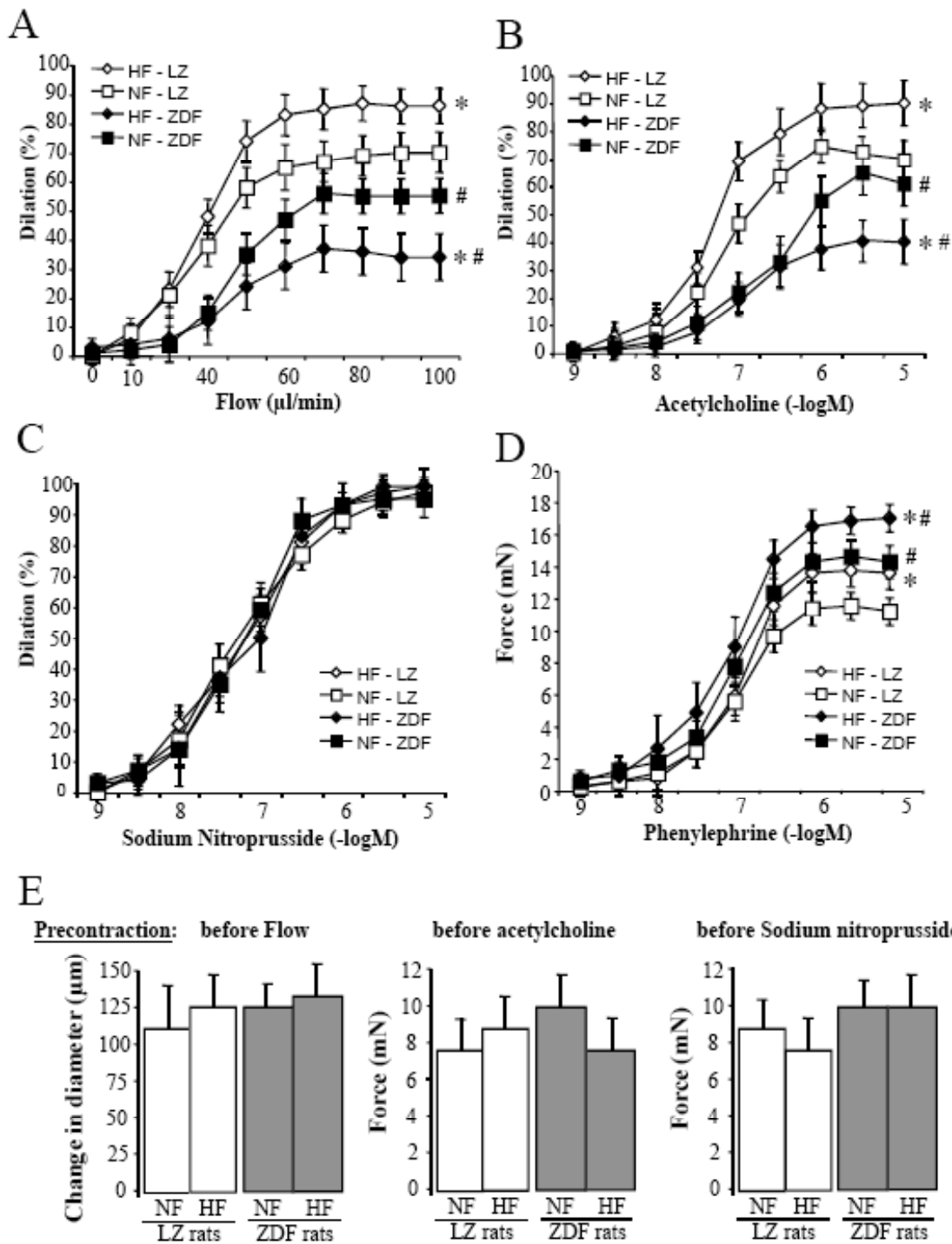


**Figure 1:** Drawing representing the model of alternative mesenteric artery ligation (A). Second order mesenteric arteries were ligated in order to increase chronically blood flow in the middle artery (HF). Equivalent arteries, located at distance are used as control or “normal flow “ (NF) arteries. The rationale of the study is present below. The production of reactive oxygen species (ROS) due to diabetes might affect the increase in diameter and the improvement of endothelium-dependent dilation induced by the chronic rise in blood flow.

Changes in diameter (B) and media cross sectional area (C) in responses to stepwise increases in pressure in HF and NF mesenteric resistance arteries isolated from control (LZ) and diabetic fatty rats (ZDF). Mean ± sem is presented (n=10 per group).

\*P<0.01, ZDF versus LZ (ANOVA for consecutive measurements).

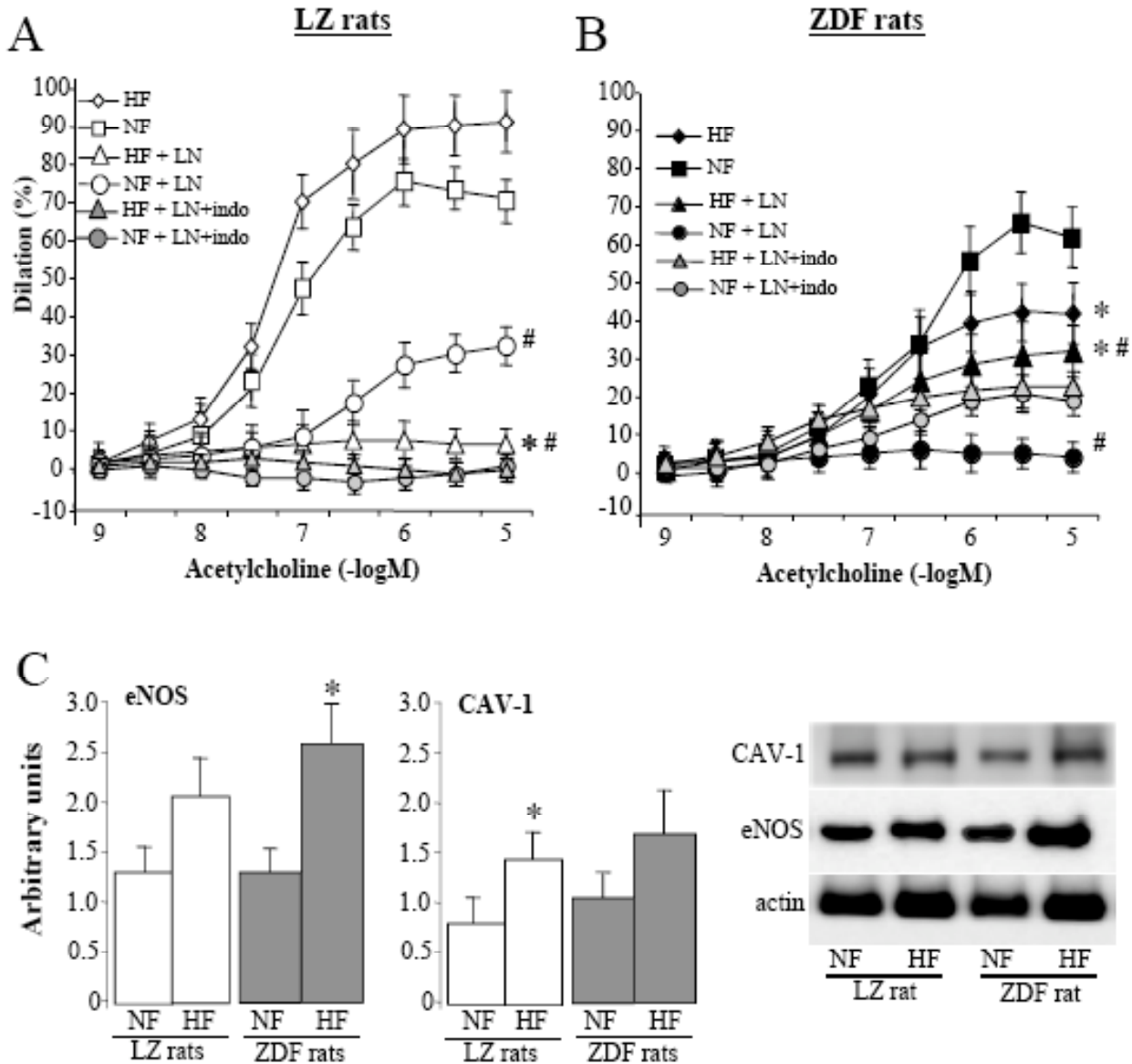
& P<0.05, NF-LZ versus NF-ZDF for a pressure of 75 mmHg.



**Figure 2:** Flow (A), acetylcholine (B) and sodium nitroprusside (C)-mediated dilation obtained in HF and NF mesenteric resistance arteries isolated from LZ and ZDF rats. Panel D: phenylephrine-induced constriction in the same arteries. Panel E: Level of precontraction obtained in arteries of the different groups before the application of flow, acetylcholine or sodium nitroprusside. Mean  $\pm$  sem is presented (n=10 per group).

#P<0.01, ZDF versus LZ rats within NF or HF groups.

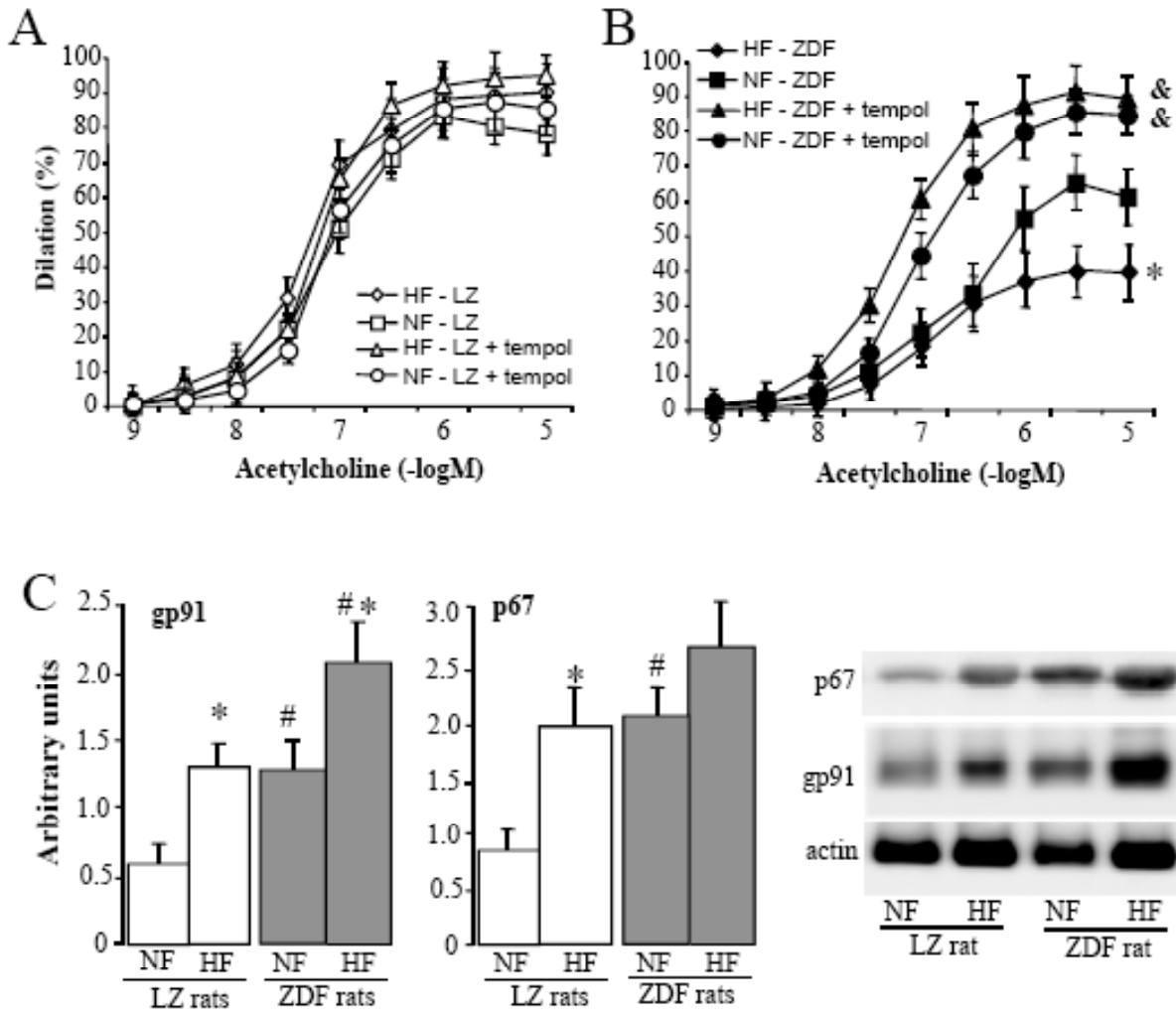
\*P<0.01, HF versus NF in each group.



**Figure 3:** Effects of eNOS inhibition (L-NAME 100  $\mu\text{mol/L}$ , LN) and COX inhibition (indomethacin 10  $\mu\text{mol/L}$ , indo) on acetylcholine-induced dilation in HF and NF mesenteric resistance arteries isolated from LZ (A) and ZDF rats (B). Panel C: eNOS and caveolin-1 (CAV-1) expression level measured in HF and NF arteries from LZ and ZDF rats. A representative blot obtained with arteries isolated from a ZDF and a LZ rat, is represented on the right side of the bargraph. Mean  $\pm$  sem is presented (n=10 per group).

<sup>#</sup>P<0.01, ZDF versus LZ rats within HF and NF groups.

\*P<0.01, HF versus NF in each group.



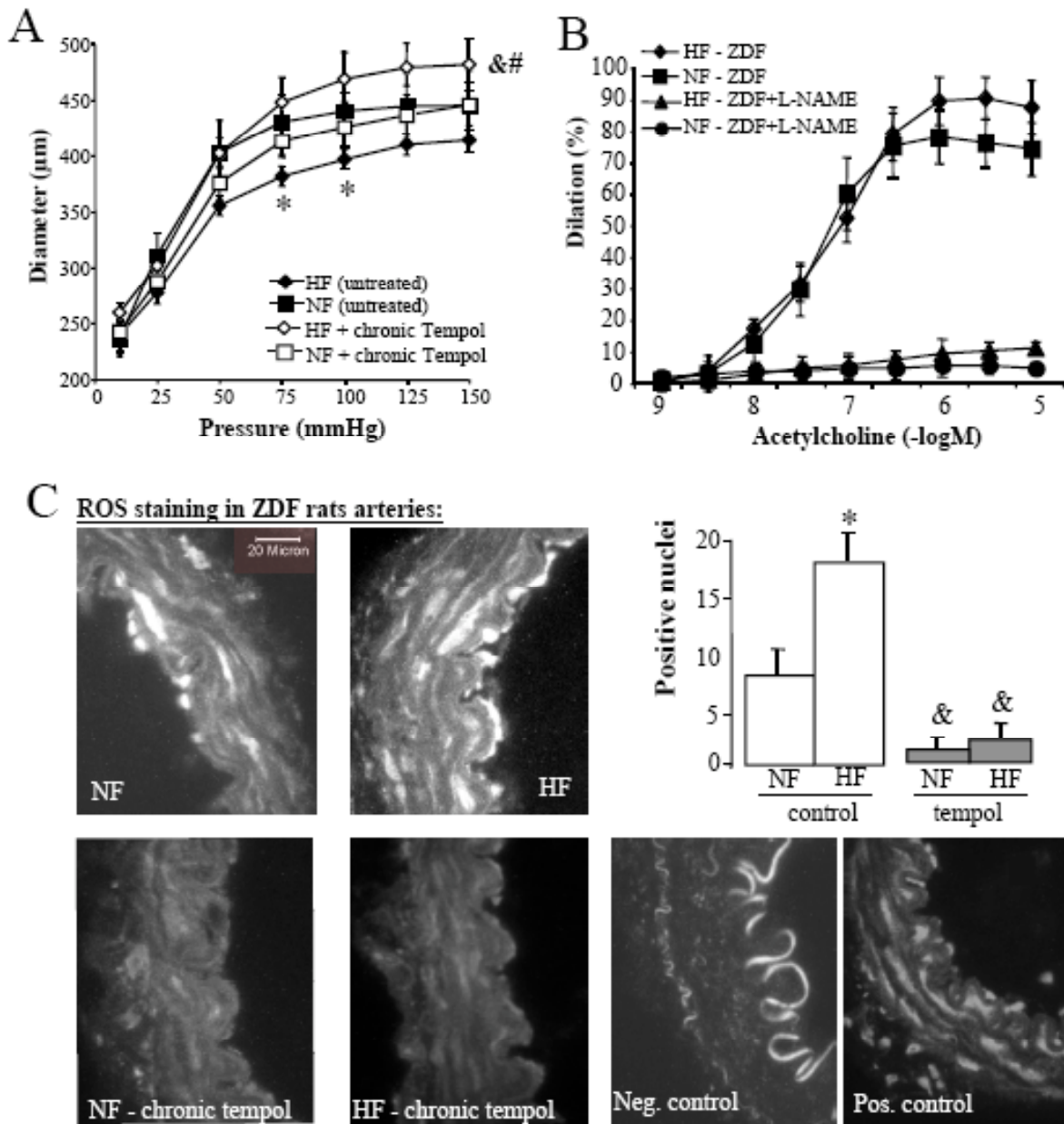
**Figure 4:** Effect of acute ROS reduction (acute tempol infusion) on acetylcholine-induced dilation in HF and NF mesenteric resistance arteries isolated from LZ (A) and ZDF rats (B). The expression level of the NADP(H)-oxidase subunit gp91 and p67 was measured by Western-blot (C). A representative blot obtained with arteries isolated from a ZDF and a LZ rat, is represented on the right side of the bargraph. Mean  $\pm$  sem is presented (n=10 per group).

&P<0.01, effect of tempol on acetylcholine-induced dilation.

\*P<0.01, HF versus NF in each group (A & B).

#P<0.01, ZDF versus LZ rats within NF or HF group (C).





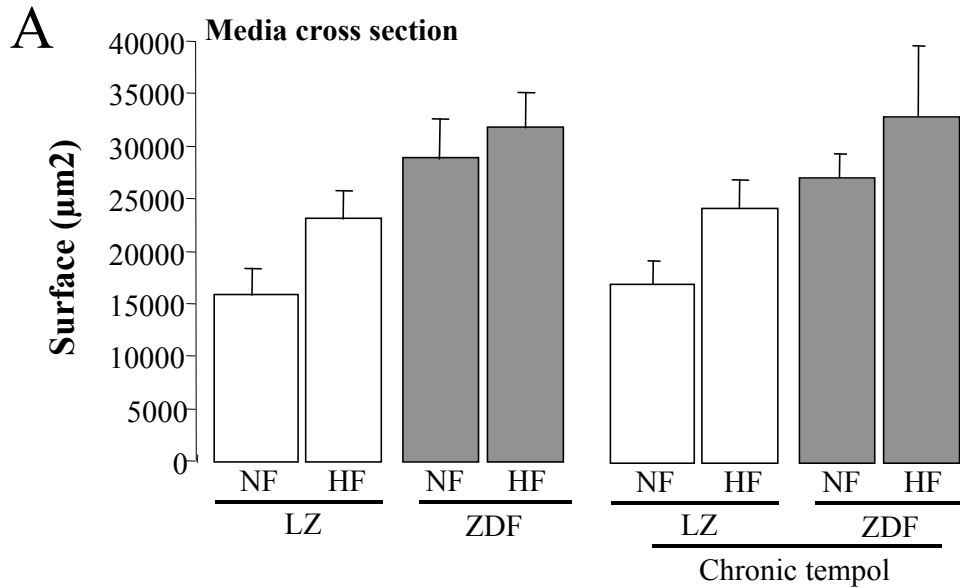
**Figure 5:** Effect of a chronic treatment of ZDF rats with tempol on passive arterial diameter (A) and acetylcholine-induced dilation (B) in HF and NF mesenteric resistance arteries. In situ detection of superoxide (ROS) using DHE staining (C) was performed in arteries and quantified (bargraph on the right). In a negative control DHE was omitted and a positive control was obtained from a rat treated with lipopolysaccharide.

&P<0.01, effect of chronic tempol.

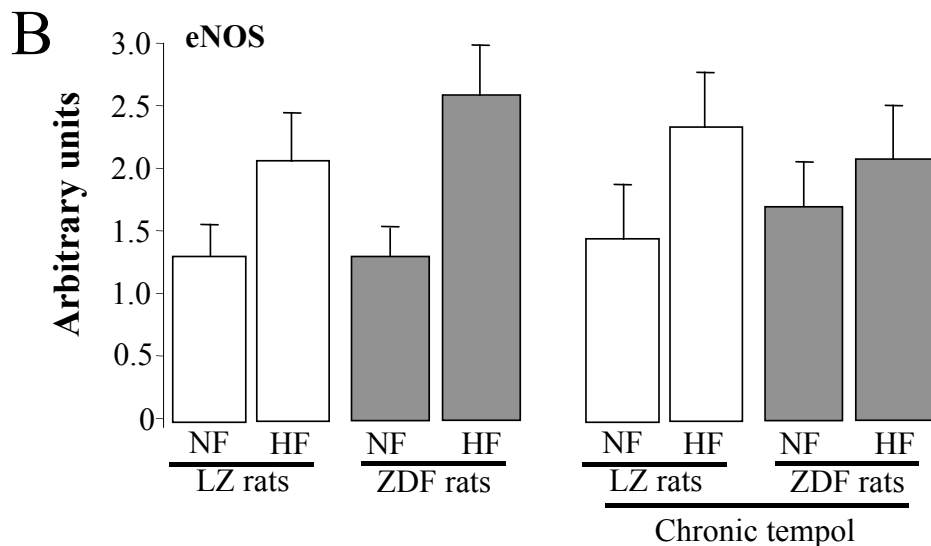
\*P<0.01, HF versus NF in each group.

#P<0.01, ZDF versus LZ rats within NF or HF group.

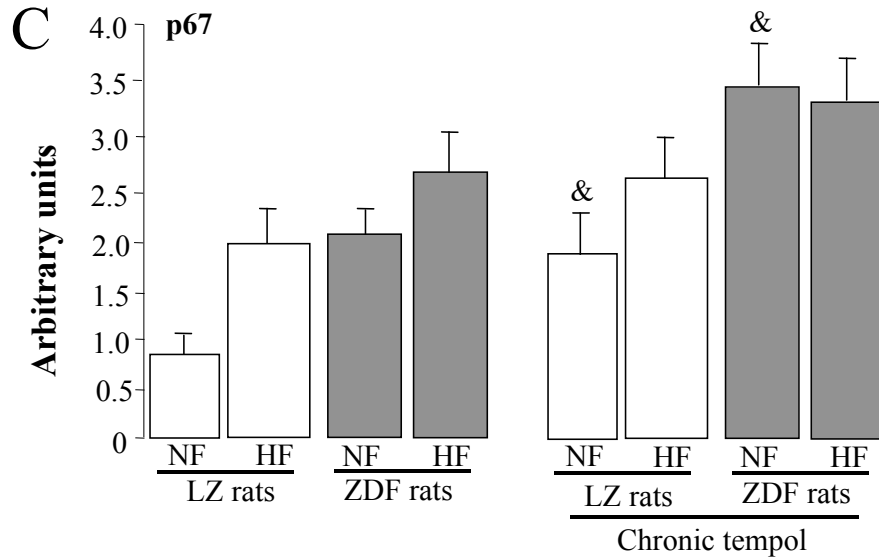
**Supplements:**



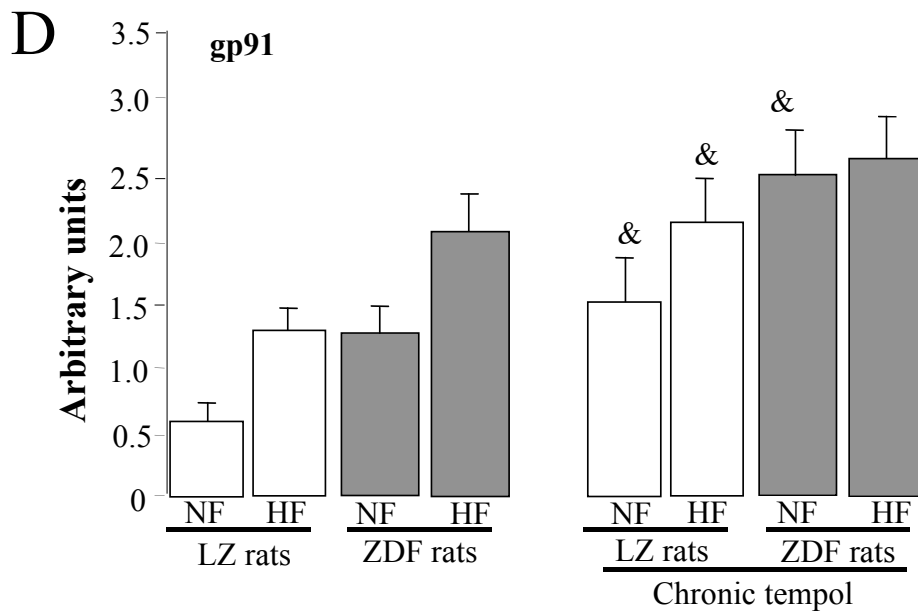
A: Changes in media cross sectional area in HF and NF mesenteric resistance arteries isolated from control (LZ) and diabetic fatty rats (ZDF) treated (right panel) or not (left panel) with tempol. Left panel is taken from figure 1. Mean  $\pm$  sem is presented (n=10 per group). NS, chronic tempol versus control (untreated).



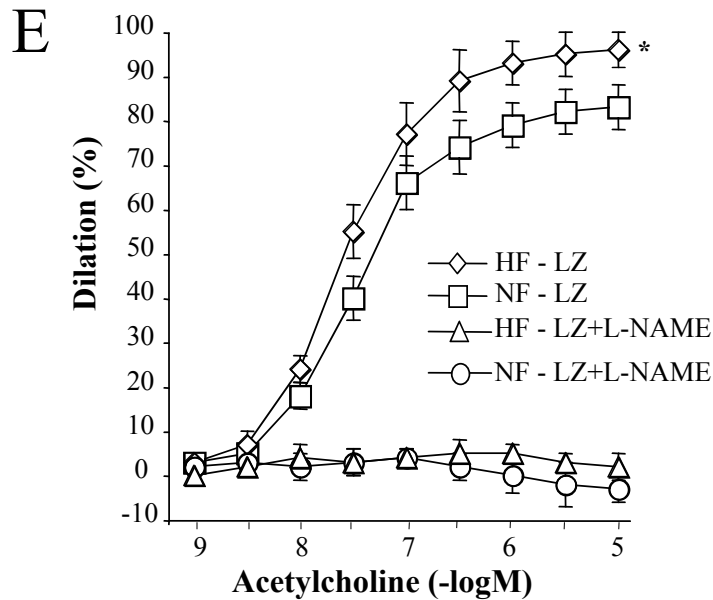
B: eNOS expression level measured in HF and NF arteries from LZ and ZDF rats treated (right panel) or not (left panel) with tempol. Mean  $\pm$  sem is presented (n=10 per group). Left panel is taken from figure 3. & P<0.05, chronic tempol versus control (untreated).



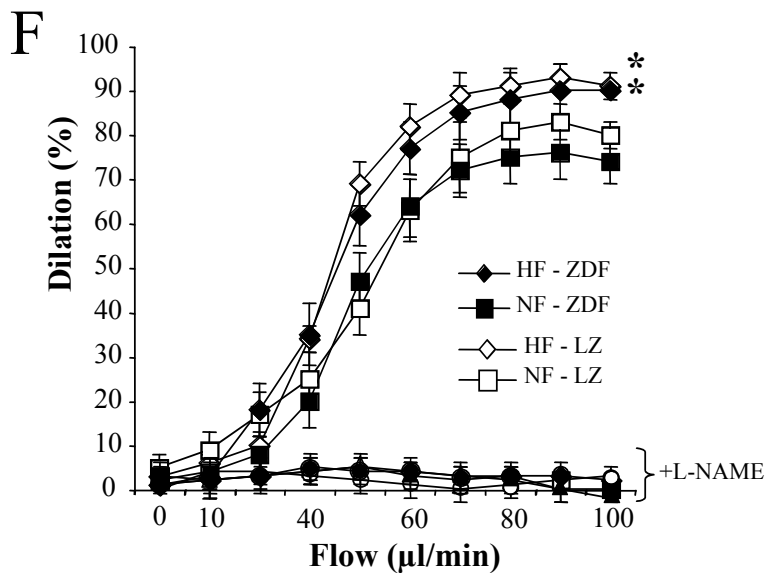
C: Expression level of the NADP(H)-oxidase subunit p67 in HF and NF arteries from LZ and ZDF rats treated (right panel) or not (left panel) with tempol. Mean  $\pm$  sem is presented (n=10 per group). Left panel is taken from figure 4. & P<0.05, chronic tempol versus control (untreated).



D: Expression level of the NADP(H)-oxidase subunit gp91 in HF and NF arteries from LZ and ZDF rats treated (right panel) or not (left panel) with tempol. Mean  $\pm$  sem is presented (n=10 per group). Left panel is taken from figure 4. & P<0.05, chronic tempol versus control (untreated).



**E:** Acetylcholine-induced dilation with and without L-NAME in HF and NF mesenteric resistance arteries isolated from LZ rats chronically treated with tempol. Mean  $\pm$  sem is presented (n=10 per group). \*P<0.05, HF versus NF.



**F:** Flow-mediated dilation with and without L-NAME in HF and NF mesenteric resistance arteries isolated from ZDF and LZ rats chronically treated with tempol. Mean  $\pm$  sem is presented (n=10 per group). \*P<0.05, HF versus NF.