Physical Training Increases eNOS Vascular Expression and Activity and Reduces Restenosis After Balloon Angioplasty or Arterial Stenting in Rats

Ciro Indolfi,* Daniele Torella,* Carmela Coppola, Antonio Curcio, Francisca Rodriguez, Antonio Bilancio, Antonio Leccia, Oreste Arcucci, Mariacristina Falco, Dario Leosco, Massimo Chiariello

Abstract—The effects of dynamic exercise on restenosis after vascular injury are still unknown. The consequences of balloon dilation–induced injury on neointimal hyperplasia, vascular negative remodeling, and reendothelialization were assessed in sedentary and trained rats. Ex vivo eNOS vascular expression and activity were investigated in carotid arteries isolated from sedentary and exercised rats. The in vivo effects of eNOS inhibition by L-NMMA on vessel wall after balloon dilation were evaluated in sedentary and exercised rats. We also investigated the effects of exercise on neointimal formation in a rat stent model of vascular injury. Compared with sedentary group, the arteries isolated from trained rats showed higher levels of eNOS protein expression and activity 7 days after balloon dilation. A significant reduction of both neointimal hyperplasia and negative remodeling was observed 14 days after balloon injury in trained compared with sedentary rats. Moreover, we demonstrated that exercise training produced accelerated reendothelialization of the balloon injured arterial segments compared with sedentary. L-NMMA administration eliminated the benefits of physical training on vessel wall after balloon dilation. Finally, a decrease of neointimal hyperplasia as well as of platelet aggregation was observed after stent deployment in trained rats compared with sedentary. In conclusion, physical exercise could favorably affect restenosis after balloon angioplasty and stenting. Increase in eNOS expression and activity might contribute to the potential beneficial effects of exercise on the vessel wall after vascular injury. (Circ Res. 2002;91:1190-1197.)

Key Words: exercise ■ restenosis ■ balloon angioplasty ■ stent ■ nitric oxide synthase

Restenosis is still an unresolved issue of percutaneous coronary interventions (PCI), although rapamycin–eluting stents recently show considerable promise for restenosis prevention.1 Restenosis after balloon angioplasty is primarily due to negative vascular remodeling and only partially to vascular smooth muscle cell (VSMC) proliferation.3 On the other hand, whereas stent deployment abolished inward vascular remodeling, in-stent restenosis (ISR) is determined by VSMC proliferation generating neointimal formation.3

The molecular pathways underlying VSMC migration and proliferation after balloon angioplasty and stenting are well known.4–6 However, the reasons for negative remodeling after balloon angioplasty are still unclear.

Endothelial damage after PCI has been associated with vascular remodeling. In fact, after balloon angioplasty and stent deployment, the vessel is almost totally endothelium-denuded.7,8 NO, synthesized by endothelial nitric oxide synthase (eNOS), is a key molecule preventing the detrimental consequences of arterial injury on the vascular wall9,10; indeed, NO inhibits VSMC migration and proliferation, platelet adhesion to the vessel wall, and stimulates endothelial cell migration and reorganization.9,10 All these actions lead to the inhibition of both vascular negative remodeling and neointimal formation after vascular injury.9,10 It is noteworthy that the beneficial effects of statins on preventing restenosis after vascular injury, both inhibiting VSMC proliferation11 and accelerating reendothelialization,8 are related to increased eNOS expression and activity.12,13

Several population-based studies demonstrated that incremental levels of regular physical activity are inversely proportional to long-term cardiovascular mortality.14 Physical
exercise preserves the endothelium-dependent NO availability increasing eNOS expression and activity on vascular wall.\textsuperscript{15,16} However, the effects of dynamic exercise on neointimal hyperplasia and arterial remodeling after vascular injury have not yet been studied.

The aims of the present study were to evaluate the effects of dynamic exercise and the involvement of exercise-mediated eNOS modulation on restenosis after experimental balloon angioplasty and arterial stenting.

Materials and Methods

Balloon Angioplasty Study Design

To assess the effects of physical training both on neointimal hyperplasia and on vascular remodeling after balloon dilation, 20 Wistar rats were randomly assigned to one of the following groups: group I, Swimming-Balloon-Angioplasty (n=10); group II, Sedentary-Balloon-Angioplasty (n=10). Experimental rat balloon angioplasty of the common carotid artery was performed using the Clowes method as previously described and well validated in our laboratory.\textsuperscript{17–20} To evaluate the role played by NO, L-NMMA was administered in drinking water (60 mg/L) to 20 rats assigned to one of the following groups: group III, Swimming-Balloon-Angioplasty+L-NMMA (n=10); group IV, Sedentary-Balloon-Angioplasty+L-NMMA (n=10). Finally, an additional 8 rats were anesthetized and underwent only to the surgical procedure without the balloon injury (Sham-Operated group). Animals in this study were purchased from Charles River, Calco, Italy, and were handled according to the animal welfare regulation of Federico II University of Naples, and the protocol was approved by the animal use committee of this institution in accordance with the animal use principles of the American Society of Physiology.

Histological Analysis

At the time of the final experiments, the animals were anesthetized and the carotid arteries removed. Both the circumference and the cross sectional area of external elastic lamina (EEL), internal elastic lamina (IEL), lumen, media, and neointima were measured and the ratios between neointima and media were calculated.\textsuperscript{17}

Arterial Remodeling

To investigate the effect of balloon dilation on the arterial remodeling, the ratio between EEL circumference (EELc) of right injured artery (EELc\textsubscript{Rig}) and EEL of left noninjured artery (EELc\textsubscript{Lig}) was calculated as arterial remodeling index (ARI).\textsuperscript{21,22} The same ratio was calculated using EEL dimensions as cross sectional area.

Immunohistochemistry for Reendothelialized Area

To measure the reendothelialized area 14 days after balloon-induced endothelium denudation, cross-sections from the approximate proximal (n=10), mid (n=10), and distal (n=10) portion of the injured artery (from sedentary and exercised animals) were stained with a polyclonal antibody for eNOS (Santa Cruz Biotechnology). Fourteen days after balloon angioplasty, the reendothelialization was expressed as the media of the percentage of positive eNOS inner surface of the entire luminal circumference in each section (from proximal, mid, and distal segments of the injured arteries) using a computerized sketching program (Morphometry-System, Bioblock Scientific).

Immunoblottings

We performed the balloon angioplasty in an additional 5 sedentary and 5 exercised animals. Because EC regeneration became significant 7 days after balloon dilation, at this time point the animals were euthanized and the injured carotid arteries were excised and immediately snap-frozen in liquid nitrogen and processed to assess eNOS and CD31 (specific for ECs) vascular expression. An additional 5 sham-operated rats were used as positive control.

eNOS Activity Assay

In carotid arteries isolated from sedentary (n=5) and exercised rats (n=5) 7 days after balloon dilation, and from sham-operated animals (n=5), the vascular eNOS activity was detected by measuring the conversion of [\textsuperscript{3}H]-arginine to [\textsuperscript{3}H]-citrulline at 37°C for 30 minutes using the commercially available eNOS assay kit (Amer- sham) as previously described.\textsuperscript{23}

Quantification of Cell Proliferation in Medial/Intimal Lesions

To differentiate VSMC proliferation in vivo, we performed the balloon angioplasty in further sedentary (n=4) and exercised (n=4) rats and in L-NMMA–treated sedentary (n=4) and exercised (n=4) rats. At 2 days after balloon angioplasty, these animals were anesthetized and the carotid arteries were carefully fixed in vivo. Monoclonal antibody against proliferating cell nuclear antigen (PCNA; DAKO) was used as a specific marker for proliferating cells as previously described.\textsuperscript{11,21}

Stent Study Design

To study the effects of physical training on neointimal hyperplasia after stent deployment, 20 Wistar rats (body weight: 500 to 520 g) were randomly assigned to one of the following groups: group I, Swimming-Stent (N=10); group II, Sedentary-Stent (N=10). In these rats, Guidant Multilink stents (4-mm length) were implanted in the right common carotid artery as previously described.\textsuperscript{11,21} The effect of stent implantation on neointimal formation was assessed 28 days later.

ADP-Induced Platelet Aggregation

Twenty-eight days after stent deployment, at the time of the final experiment, a blood sample was taken from the left carotid artery in 8 rats in Sedentary-Stent group and in 8 rats of Swimming-Stent group, and ADP-induced platelet aggregation was evaluated.

Exercise Training

In all the swimming groups of the two protocols (Angioplasty or Stenting), training consisted of 90 minutes (45 minutes×2; 8:00 AM and 8:00 PM) swimming per day (6 days weekly) performed in a 150-L water tank with water temperature between 27°C to 28°C. In the Sedentary groups, the animals did no exercise. In the swimming groups, rats were trained for 14 days before vascular injury, and during 14 and 28 days after balloon injury and stenting, respectively.

Statistical Analysis

Statistical analysis was performed as described in the expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Results

Rat Balloon Angioplasty Protocol

Immunoblottings

A positive immunoreactive eNOS protein expression was detected in the arteries of sham-operated animals (Figures 1A and 1B). In sedentary group, balloon dilation caused a significant reduction of eNOS expression in the right injured arteries compared with left uninjured arteries and compared with carotid vessels isolated from sham rats (Figures 1A and 1B). In the right injured arteries of exercised animals, we observed a slight reduction of eNOS expression compared with the uninjured left arteries (Figures 1A and 1B). However, both the right and left carotid arteries isolated from exercised rats showed a remarkable increase of eNOS expression compared with the right and left carotid vessels isolated from sedentary and sham animals (Figures 1A and 1B). A key
question arose in relation to whether the level of eNOS protein expression 7 days after balloon dilation reflects faster reendothelialization in the exercise-trained animals versus sedentary. To this aim, we performed Western blotting analysis for CD31. Seven days after balloon injury, the expression of CD31 was severely reduced in the injured arteries of the sedentary animals compared with the contralateral left uninjured vessels and to the carotid arteries isolated from sham rats, reflecting loss of ECs (Figures 1A and 1C). On the other hand, CD31 in injured arteries isolated from exercised animals increased compared with injured vessels from sedentary, even if it was still less than in sham-operated animals and in uninjured left arteries (Figures 1A and 1C). Therefore, although EC number seems to return more rapidly to normal in exercised rats compared with sedentary, the difference in eNOS expression observed between trained and sedentary animals is most likely due to an actual eNOS induction rather than only to a simple increase in EC number. The actual increased eNOS expression in arteries from exercised rats was also suggested by the net increase in eNOS vascular expression in the un.injected carotid artery of these animals compared with the uninject ed carotid artery of sedentary and sham animals (Figures 1A and 1B).

**eNOS Activity Assay**

Seven days after balloon dilation, eNOS activity in the injured right carotid arteries isolated from sedentary animals showed a 4-fold reduction compared both to the contralateral left carotid vessels and arteries from sham rats (Figure 2). In trained animals, eNOS activity was only slightly reduced in the injured carotid arteries compared with the uninjured arteries (Figure 2). However, eNOS activity in the right injured and left uninjured vessels isolated from exercised showed an absolute significant increase compared both to the vessels from sham and sedentary animals (Figure 2).

**Quantification of In Vivo Cell Proliferation in Injured Carotid Vessels**

PCNA-stained nuclei were observed in the neointimal and medial layers of blood vessels at 2 days after vascular injury...
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Morphological Findings in the Balloon Angioplasty Protocol

<table>
<thead>
<tr>
<th></th>
<th>Seden Swimm</th>
<th>Seden+L-NA Swim+L-NA</th>
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<td>N/M ratio</td>
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<tr>
<td>Lumen diameter, mm</td>
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<td>Lumen CSA, mm²</td>
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<tr>
<td>PCNA index 2 days</td>
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</table>

Seden indicates Sedentary group; Swimm, Swimming group; Seden+L-NA, Sedentary+L-NMMA group; Swim+L-NA, Swimming+L-NMMA group; R, Right Injured carotid artery; L, Left uninjured carotid artery; Neointim. CSA, Neointimal cross sectional area; N/M Ratio, Neointima CSA/media CSA ratio; Lumen CSA, Lumen cross sectional area; Lumen diameter, Minimal lumen diameter; EEEl, External elastica lamina area; EEElc, External elastica lamina circumference; AR1, Arterial remodeling index (EEEl area/EEElc area); AR2, Arterial remodeling index (EEElc/EEElc); PCNA index 2 days, Percent positive PCNA cells at 3 days after balloon injury.

Effects of Physical Training on Neointimal Hyperplasia After Experimental Balloon Angioplasty

Fourteen days after balloon angioplasty, physical training reduced neointimal tissue in exercised rats compared with sedentary animals (Figure 4; Table). Long-term administration of L-NMMA, inhibiting eNOS in vivo activity, in exercised animals produced a neointimal mass similar to the sedentary animals after balloon angioplasty (Figure 4; Table). We addressed the time course of the neointimal formation in vivo, measuring the neointimal area and neointima/media ratio at 2 days and 7 days after balloon dilation in the arteries isolated, respectively, from the animals used for the PCNA index (n=16) and for the immunoblotting analysis (n=20). Interestingly, physical training reduced significantly neointimal area and neointima/media already at 7 days after balloon angioplasty compared with sedentary (online Table 1, which can be found in the online data supplement available at http://www.circresaha.org).

Effects of Physical Training on Vascular Remodeling After Experimental Balloon Angioplasty

Fourteen days after balloon dilation, digital planimetry revealed vascular negative remodeling in the Sedentary-Balloon-Angioplasty Group compared with sham animals (Figure 5; Table). Physical training significantly prevented vascular negative remodeling (Figure 5; Table). The effect of physical training in preventing negative remodeling was already significant at 7 days after balloon dilation (online Table 1). Furthermore, in the exercised animals, we consistently observed a significant increase in EEL circumference both in right injured and in left noninjured arteries compared with sedentary (online Table 1 and online Figure 1). These results demonstrate that physical training not only prevents negative remodeling in balloon-injured arteries but also causes an outward positive remodeling in normal not injured vessels. This phenomenon might be related to eNOS activity because long-term L-NMMA administration prevented the beneficial effects of physical training on vascular remodeling after experimental balloon angioplasty (Figure 5; Table).

Effects of Exercise Training on Reendothelialization

Fourteen days after balloon angioplasty, we observed that exercise training produced accelerated reendothelialization in all the analyzed arterial segments (proximal, middle, and distal) compared with the arteries isolated from sedentary animals. Indeed, in the swimming group, the new endothelium on neointima layer circumference was nearly complete proximally and distally of the balloon-injured segment (respectively, 90.4±5.5% and 94.4±4.6%) (Figure 6); in contrast, the reendothelialized circumference was limited at the same segments in the sedentary group (proximally, 75.5±3.4; P<0.01 versus swimming group; distally, 82.5±4.7; P<0.01 versus swimming group) (Figure 6). On the other hand, in the exercised animals, an incomplete reendothelialized circumference was observed in the middle portion of the injured segment (44.5±6.4%) that was still higher compared with sedentary rats in the same carotid segment (20.5±5.1%; P<0.01 versus swimming group) (Figure 6). L-NMMA
administration to the exercised animals determined a reendothelialization similar to sedentary (proximal, 74.0 ± 6.10; \( P < 0.001 \) versus sedentary; middle, 18.5 ± 4.5; \( P = \text{NS} \) versus sedentary; distal, 77.2 ± 3.80; \( P = \text{NS} \) versus sedentary) (Figure 6), demonstrating the key role played by eNOS.

Rat Stent Protocol

**Effects of Physical Training on Neointimal Hyperplasia After Arterial Stenting**

In sedentary animals (sedentary-stent group; \( n = 10 \)), 28 days after arterial stenting a significant neointimal hyperplasia and neointima/media ratio were observed (0.486 ± 0.069 mm² and 1.727 ± 0.194) (Figures 7A and 7B). Physical training (swimming-stent group; \( n = 10 \)) reduced the neointima hyperplasia (0.211 ± 0.046 mm²; \( P < 0.001 \) versus sedentary-stent group) and the neointima/media ratio (0.794 ± 0.232; \( P < 0.001 \) versus Sedentary-Stent group) (Figures 7A and 7B).

**ADP-Induced Platelet Aggregation**

Figure 7C shows that physical training reduced ADP-induced platelet aggregation (AIPA) in the exercised animals (AIPA, 19±2%) compared with sedentary (AIPA, 33±3%; \( P < 0.001 \) versus exercised animals).

### Discussion

The major findings of the present study are as follows: (1) physical training upregulates eNOS protein expression and activity in the arterial wall; (2) regular exercise affects favorably arterial remodeling, significantly inhibits neointimal hyperplasia, and accelerates reendothelialization after experimental balloon angioplasty; and (3) in the exercised animals, a significant reduction of the neointimal tissue growth and a reduced platelet aggregation were observed after stenting.

### Role of Physical Training on the Mechanisms Responsible for Restenosis After Balloon Angioplasty and Stenting

Exercise training has assumed a major role in both the primary and secondary prevention of coronary artery disease (CAD).\(^{24,25}\) One of the clues of the mechanisms leading to these benefits of exercise is the increased bioavailability of NO in the arteries of trained men.\(^{15}\) In particular, previous data reported that exercise increases eNOS expression and activity in vascular wall.\(^{16,25}\) NO, synthesized by eNOS, is involved in the regulation of vascular remodeling and neointimal formation after vascular injury.\(^{10}\) Endothelial NOS knockout mice show a worsening vascular remodeling in response to a decrease in blood flow.\(^{26}\) Moreover, these animals also exhibit an exaggerated neointimal hyperplasia in response to vascular injury.\(^{27}\) In vivo eNOS overexpression, by gene transfer in the injured carotid artery, yielded a significant reduction of neointimal formation.\(^{28}\)

Human studies have recently shown that negative remodeling explains up to 70% of late lumen loss after balloon angioplasty.\(^{3}\) In-stent restenosis, on the other hand, is entirely due to VSMC proliferation.\(^{3}\) It should be pointed out that the present study demonstrated that a program of physical training, increasing arterial eNOS expression and activity, inhibits neointimal hyperplasia after experimental balloon angioplasty and prevents negative inward vascular remodeling. The data of the present study also demonstrated a significantly lower index of PCNA-positive cells after injury to the arteries of exercised animals compared with the arteries of sedentary animals. This finding suggests that the decrease of neointimal tissue was at least in part due to an antiproliferative effect of the increased eNOS activity. Indeed, eNOS inhibition with L-NMMA administration prevented the beneficial effects of exercise on neointimal hyperplasia. The pure antiproliferative effect of exercise was also demonstrated in the stent model in which a reduction of neointimal tissue was observed in exercised animals compared with sedentary.

### Effects of Physical Training on Reendothelialization

Interventional strategies such as balloon angioplasty or coronary stent implantation invariably result in a marked degree of vascular injury.\(^{7}\) In the hours after experimental angioplasty, endothelial cells rapidly enter the replication cycle to restore endothelial continuity.\(^{7}\)

The loss of the endothelial monolayer is associated with a variety of deleterious consequences such as thrombus forma-
tion, neointimal thickening, and negative vascular remodeling.\textsuperscript{7}

It should be pointed out that all the tools used to prevent restenosis, including stent-based drug delivery, might delay maturation and normal endothelial function, thus increasing the potential for a late thrombotic event. Indeed, a recent study evaluated the first clinical experience with Taxol-eluting stents for ISR, and the encouraging positive results in terms of low MACE rate at 6 months are almost eliminated at 12 months.\textsuperscript{29} The delay of reendothelialization is an important issue that can limit the beneficial effect of eluting stents.

Consequently, achieving the complete reendothelialization of injured vessel, after both balloon angioplasty and stenting with a regenerated endothelium showing normal morphological characteristics and functions may favorably affect restenosis.

It should be noted that NO, produced by eNOS, stimulates endothelial cell migration and reorganization after vascular injury in vivo,\textsuperscript{10} and in our study, we demonstrated an increased eNOS vascular expression and activity in animals enrolled in a program of physical exercise. Microscopic assessment of reendothelialization was performed using eNOS endothelial immunostaining on several cross sections. We observed a greater degree of reendothelialization at all the examined arterial segments in the exercised animals compared with sedentary after balloon dilation.

Therefore, accelerated reendothelialization, secondary to eNOS increased expression and activity, may contribute to the effects of exercise on restenosis after vascular injury.

**Physical Training and Platelet Aggregation**

NO plays a critical role in maintaining vascular homeostasis, including inhibition of platelet aggregation and adhesion to the vessel wall.\textsuperscript{9} It has been demonstrated that acute exercise can lead to increased platelet activity, especially in sedentary individuals,\textsuperscript{30} but regular exercise may eliminate or reduce this response.\textsuperscript{31} In the present study, the animals, enrolled in a program of regular physical training, showed a decreased platelet aggregation that could partially explain the reduced neointimal hyperplasia after stent deployment in these animals.

Recent data has shown that neointimal hyperplasia variations after stent deployment at 6-month follow-up are inversely related to the relative shear stress distribution.\textsuperscript{32} It is also known that shear stress through NO availability affects platelet aggregation.\textsuperscript{9} In our study, exercise significantly increased carotid blood flow velocity after stenting (online Table 2), suggesting a hemodynamic mechanism underlying in-stent neointimal hyperplasia reduction in exercised animals.

**Limitations and Study Implications**

Because in many patients the endothelial function is chronically depressed, exercise-induced increases in eNOS might be
difficult to achieve due to symptom limitation before PCI. However, a different degree of endothelial function might be present in CAD patients, and eNOS activity may still increase with exercise in many patients. Regarding the possibility to perform exercise before the PCI, it should be pointed out that acute coronary syndrome (ACS) usually occurred suddenly. Some of these subjects are already engaging physical training (jogging, etc) before ACS, and after percutaneous revascularization a regular exercise program could be continued. On the other hand, for stable patients with coronary artery disease, exercise can be safely performed without achieving the ischemic threshold. Nevertheless, further clinical trials should be performed in order to demonstrate this hypothesis.

The physiologically most important stimuli activating eNOS is the fluid shear stress that amplifies eNOS activity and expression.30 Shear stress modulates vascular remodeling after balloon angioplasty and is also an important component of exercise affecting vascular NO concentrations through eNOS increased expression and activity.25 Therefore, the increased vascular eNOS expression and the resulting inhibition of both neointimal formation and negative remodeling observed in trained rats in the present study could suggest a hemodynamic mechanism modulating these effects. To this regard, 14 days after balloon angioplasty, Doppler probe analysis revealed that physical training increased carotid blood flow velocity (CBFV) compared with sedentary (online Table 3 and online Figure 2). Combining these values of the CBFV with the histological measurements of the vessel lumen areas, we derived the actual carotid blood flow and wall shear rate (WSR), which were increased in the swimming groups compared with sedentary (online Table 3 and online Figure 2).

An important limitation of these results was that only carotid blood flow velocity was measured in vivo and not actual blood flow. Indeed, the values of this parameter was obtained combining in vivo CBFV parameters with ex vivo histological findings that cannot be interpreted as absolute values but only looking at them as directional changes. Therefore, extreme caution should be taken in account when interpreting them and the link between exercise, shear stress, and vascular remodeling should be carefully addressed in further studies using larger animals in which arterial dimensions can be measured in vivo with IVUS.

Summary

The data of the present study strongly suggest a beneficial role of physical exercise on vascular negative remodeling, smooth muscle cell proliferation, and reendothelialization after vascular injury. Nitric oxide synthase seems to play an important role on these exercise-induced beneficial effects. However, further clinical studies should be performed in order to evaluate the effective impact of physical training on restenosis after angioplasty or stenting.

Acknowledgments

This work was supported by the Department of Clinical Medicine, Cardiovascular and Immunological Sciences, Federico II University, Naples, Italy, and the Genecor Foundation, a nonprofit Cardiovascular Association, Naples, Italy.

References


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Circ Res. 2002;91:1190-1197; originally published online November 7, 2002;
doi: 10.1161/01.RES.0000046233.94299.D6
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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MATERIALS and METHODS

Hemodynamic Measurements

Arterial pressure and heart rate were measured directly by an invasive technique in all groups at the moment of the final experiment. To assess carotid blood flow velocity (CBFV), a rigid circumferential probe was applied around the vessel 14 days after balloon injury on the carotid artery. As it is well known that in the rat model of carotid balloon injury the biggest amount of neointima tissue is in the middle portion of the injured vessel, in order to avoid jeopardizing this neointima portion, the external probes were applied distally to this injury site. Since the rigid probe fixed the external circumference of the vessel to the probe internal diameter, subtracting the local wall area (which also depends on the amount of intimal growth, i.e. the media and neointimal area) from the probe enclosed area, the lumen diameter was derived and applying this value to the in vivo measurements of the CBFV, the actual blood flow (assuming a parabolic flow profile) was estimated as follows:

$$\text{CBF} = (V) \times (\pi r^2) \times 0.001$$

where CBF is the volume of carotid blood flow (nl/s), V is the CBFV (mm/s), and r is the vessel radius (µm). We applied this flow to the lumen dimensions measured at the site distal to the injury from histology to obtain insight in the local shear stress values. In detail, wall shear rate (WSR; sec⁻¹) was calculated (assuming a constant blood viscosity) as outlined by Koller and Kaley (17) as follows:

$$\text{WSR} = 8 \times (V \times D^{-1})$$

where V is the blood flow velocity in the carotid vessel and D is the vessel diameter obtained by subtracting the local wall area (neointima and media areas) from the probe enclosed area (=0.400 mm²).

Histological Analysis

At 2 (from animals used for assessing VSMC proliferation in vivo), 7 (from animals used for immunoblottings) and 14 days after balloon angioplasty, the carotid arteries were fixed by
perfusion at 120 mmHg with 100 ml of phosphate-buffered saline (PBS; pH 7.2), followed by
80 ml of prepared PBS containing 4% paraformaldehyde via a large cannula placed in the left
ventricle. The carotid arteries were removed and a total of 18 cross-sections were cut (each 6
µm thick) from the approximate proximal (n=6), mid (n=6) and distal (n=6) portion of the
artery. Both the circumference and the cross sectional area of external elastic lamina (EEL),
internal elastic lamina (IEL), lumen, media and neointima were measured and the ratios
between neointima and media were calculated.

The analysis of histological features was performed at different arterial levels to make sure to
select the site of minimal lumen diameter. Then, the reported quantitative measurements
were relative to the segments that exhibited the greatest amount of neointimal tissue.

Immunohistochemistry for reendothelialized area

To assess the presence of endothelial cells at the luminal surface of the vessel wall,
endothelial immunostaining was performed in the animals sacrificed for histological analysis.
Cryosections were incubated with peroxidase blocking reagent, rinsed in PBS for 10 min and
in 10% horse serum in PBS for 10 min. A mouse prepared polyclonal primary antibody to
eNOS (Santa Cruz Bio, Santa Cruz, CA), diluted to 1:20 in PBS was incubated at 37°C
overnight. After extensive washing, anti-mouse biotinylated secondary antibody was applied
for 1 h. After washing in PBS, sections were incubated for 1 h in a solution of avidin-biotin-
peroxidase preformed complex (ABC Vector-Vectastain kit, Vector laboratories Inc.,
Burlingame, CA). Ten arterial cryosections selected in three of the predefined portions
(proximal, middle and distal) were analyzed. The percent endothelialization in each section
was measured with use of a computerized sketching program (Morphometry-System,
Bioblock Scientific).

Immunoblottings

The level of expression of the eNOS and CD31 proteins was analyzed by Western blot.
Carotid tissues were pulverized and solubilized in Laemmli buffer containing 2-
mercaptoethanol, and proteins were separated in denaturing SDS-10% polyacrylamide gels (75 µg/lane). Proteins were then blotted into nitrocellulose (Immobilon-P, Millipore). Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/l Tris - HCl, 137 mmol/l NaCl, 0.1% Tween 20). Western blot analysis were performed with a monoclonal antibody against eNOS (BD Transduction Laboratories) and a goat polyclonal CD31 antibody (Santa Cruz Bio, Santa Cruz, CA). Blots were incubated with the first antibody (1:100) for 3 h at room temperature and, after extensive washing in TBS-T, with the second antibody (horseradish peroxidase-conjugated antimouse and anti-goat immunoglobulins, Santa Cruz Bio, Santa Cruz, CA) at a dilution of 1:1.000 for 45 minutes. Specific eNOS proteins were detected by enhanced chemiluminescence (Amersham) and evaluated by densitometry (Molecular Dynamics). Prestained protein markers were used for molecular mass determinations (Bio Rad). The monoclonal antibody used for eNOS detection specifically recognizes eNOS and does not cross-react with the inducible or neuronal NOS isoforms. To compare NOS and CD-31 expression with the expression of another protein, we analyzed the expression of α-tubulin by Western blot using α-tubulin monoclonal antibody (Santa Cruz Bio, Santa Cruz, CA). For this purpose, a parallel gel with identical samples was run, and after blotting onto nitrocellulose, the Western blot analysis was performed with the α-tubulin monoclonal antibody (1:200). eNOS was detected as a 140-kDa band, CD31 as a 130-kDa band, and α-tubulin as a 52-kDa band.

**Determination of endothelial NOS activity**

Frozen tissues were pulverized in a solution containing 320 mM sucrose, 50 mM Trizma base, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml aprotinin brought to pH 7 with HCl. The pulverized tissues were centrifuged at 12,000 g for 20 min (4°C). NO formation was measured in the supernatant by the rate of conversion of radiolabeled L-[14C]citrulline from L-[14C]arginine (Amersham). Tissue extracts were
incubated in a pH 7 buffer (100 µl) containing (in mM) 50 potassium dihydrogen orthophosphate, 60 L-valine, 0.12 -nicotinamide adenine dinucleotide phosphate, 1.2 L-citrulline, 1.2 magnesium chloride, 0.24 calcium chloride, 0.024 L-arginine, and 0.002 L-[U-14C]arginine (297 mCi/mmol). Because NOS is maximally active only in a narrow pH range ~7.5 (13), both the pulverized tissues and the incubation buffers were brought to pH 7 with HCl before being used. The NOS activity was expressed in pmol/min/g.

Stent deployment and Histological Analysis

At the time of stent implantation, 200 U of heparin was injected. No other antigoagulant or antiplatelet therapy was give either before and after stent deployment. We implanted the ACS Multilink stents (Guidant Corporation, Indianapolis, IN), 7 cell, 4 mm of length. The stent was crimped on a 1.5 balloon catheter Worldpass Cordis (Cordis Corporation, Miami, FL) inflated at 10 ATM for 60 seconds to expand the stent and to optimize strut apposition against the arterial wall. The angioplasty balloon catheter loaded with the stent was introduced using a dissecting microscope (LEICA, GZ4) through the right external carotid artery into the common carotid artery and then inflated (11, 21).

Twenty-eight days after stent deployment, the arteries were dehydrated and cleared, they were infiltrated and embedded in a combination of polymethylmethacrylate (PMMA), n-butyl phthalate and benzoyl peroxide. A tungsten carbide knife was used to obtain 6 µm sections from the middle of the stented arteries. Modified hematoxylin-eosin and elastin stains were used to stain the plastic-embedded stented segments. Quantitative vessel injury severity and neointimal response were derived from elastic van Gieson-stained sections. An experienced, blinded investigator performed the light microscopic examinations. Morphometric analysis of the arterial images acquired onto a PC computer from a Leitz microscope using a JVC camera (TK-C1380) was performed using NIH image software.
Statistical Analysis

All data are shown as mean ± SEM. Statistical analysis between groups was performed by analysis of variance (ANOVA) using a SPSS 10.0 program. When a significant overall effect was detected, Turkey's test was applied to compare single mean values. A p value < 0.05 was considered significant.
RESULTS:

*Hemodynamic Parameters*

Compared to the sham operated animals, in the sedentary animals, 14 days after balloon angioplasty, Doppler probes analysis revealed a reduction of CBFV in the injured common carotid artery (table 3, figure 2). Physical training increased CBFV compared to sedentary (table 3, figure 1). Chronic L-NMMA administration reduced this positive effect of physical training (table 3, fig. 2). We derived the lumen diameter by subtracting the local wall area (i.e., media and neointima areas measured at the site distal to the injury and reported in the table IV) from the probe enclosed area (=400 mm²) in order to estimate the actual carotid blood flow (CBF) that was reduced in the Sedentary and increased in the Swimming Groups (table 3, figure 2). Also in this case, L-NMMA abolished the effects of physical training (table 3, figure 2). It should be pointed out that even the wall shear rate values (WSR) had the same profile of the CBF (table 3, figure 2).
FIGURE LEGENDS:

**Figure 1**
Bars representing external elastic lamina circumference (EELc) of right and left common carotid arteries after balloon injury from: Sedentary, Swimming, Sedentary + L-NMMA and Swimming + L-NMMA Groups.

(*p<0.01 vs. relative EELc_{Left}; #p<0.01 vs. all EELc_{Left}; §p<0.01 vs. all EELc_{Right}).

**Figure 2**
Carotid Blood Flow Velocity (CBFV; mm/sec), Carotid Blood Flow volume (CBF; nl/s) and Wall Shear Rate (WSR; sec^{-1}) 14 days after balloon angioplasty in the injured common carotid artery isolated from: rats subjected to only balloon injury (SEDEN); trained rats (SWIMM); L-NMMA treated rats subjected to only balloon injury (SEDEN + L-NMMA); trained rats treated with L-NMMA (SWIMM + L-NMMA). Bar graft include also the CBFV, CBF and WSR in uninjured carotid arteries from sham operated animals (SHAM) as baseline controls.

(*p<0.01 vs. all; **p<0.01 vs. all except for SWIMM+L-NA).
<table>
<thead>
<tr>
<th></th>
<th>SEDEN</th>
<th>SWIMM</th>
<th>SEDEN+L-NA</th>
<th>SWIMM+L-NA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n=4</td>
<td>n=4</td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td>Neoint. CSA&lt;sub&gt;R&lt;/sub&gt; (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.021±0.003</td>
<td>0.017±0.005</td>
<td>0.023±0.006</td>
<td>0.022±0.005</td>
</tr>
<tr>
<td>N/M&lt;sub&gt;R&lt;/sub&gt; Ratio</td>
<td>0.162±0.019</td>
<td>0.122±0.022</td>
<td>0.177±0.060</td>
<td>0.175±0.064</td>
</tr>
<tr>
<td>EEL&lt;sub&gt;R&lt;/sub&gt; Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.454±0.028</td>
<td>0.460±0.029</td>
<td>0.449±0.008</td>
<td>0.454±0.021</td>
</tr>
<tr>
<td>EEL&lt;sub&gt;L&lt;/sub&gt; Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.457±0.025</td>
<td>0.462±0.019</td>
<td>0.452±0.020</td>
<td>0.458±0.023</td>
</tr>
<tr>
<td>ARI</td>
<td>0.993±0.026</td>
<td>0.996±0.018</td>
<td>0.993±0.016</td>
<td>0.991±0.010</td>
</tr>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>Neoint. CSA&lt;sub&gt;R&lt;/sub&gt; (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.103±0.011</td>
<td>0.059±0.014*</td>
<td>0.108±0.014</td>
<td>0.099±0.013</td>
</tr>
<tr>
<td>N/M&lt;sub&gt;R&lt;/sub&gt; Ratio</td>
<td>0.785±0.150</td>
<td>0.413±0.150*</td>
<td>0.821±0.091</td>
<td>0.784±0.211</td>
</tr>
<tr>
<td>EEL&lt;sub&gt;R&lt;/sub&gt; Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.435±0.019*</td>
<td>0.500±0.012*</td>
<td>0.433±0.020*</td>
<td>0.441±0.030*#</td>
</tr>
<tr>
<td>EEL&lt;sub&gt;L&lt;/sub&gt; Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.460±0.025</td>
<td>0.507±0.019*</td>
<td>0.458±0.018</td>
<td>0.464±0.023</td>
</tr>
<tr>
<td>ARI</td>
<td>0.946±0.016</td>
<td>0.986±0.008*</td>
<td>0.945±0.012</td>
<td>0.950±0.011</td>
</tr>
</tbody>
</table>

**SEDEN**= Sedentary group  
**SWIMM**= Swimming group  
**SEDEN+L-NA**= Sedentary +L-NMMA group  
**SWIMM+L-NA**= Swimming+L-NMMA group  
**R**= Right Injured Carotid Artery  
**L**= Left Uninjured Carotid Artery  
**Neoint. CSA**= Neointimal Cross Sectional Area  
**N/M Ratio**= Neointima CSA/Media CSA Ratio  
**EEL Area**= External Elastica Lamina Area  
**ARI**= Arterial Remodeling Index= EEL<sub>R</sub> Area/EEL<sub>L</sub> Area  
**PCNA Index 2 Days**= % positive PCNA cells at 3 days after balloon injury

*p<0.03 vs Seden, Seden+L-NA, Swimm+L-NA  
#p<0.03 vs relative EEL<sub>L</sub> Area
Table 2: Additional morphologic and hemodynamic results in the stent protocol

<table>
<thead>
<tr>
<th></th>
<th>SEDEN</th>
<th>SWIMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEL Area (mm(^2))</td>
<td>1.958±0.098</td>
<td>1.984±0.062#</td>
</tr>
<tr>
<td>Lumen CSA (mm(^2))</td>
<td>1.189±0.158</td>
<td>1.481±0.075*</td>
</tr>
<tr>
<td>Lumen Diameter (mm)</td>
<td>0.379±0.050</td>
<td>0.472±0.024*</td>
</tr>
<tr>
<td>CBFV(mm/sec)</td>
<td>15.2±1.7</td>
<td>31.5±2.5*</td>
</tr>
</tbody>
</table>

EEL Area= External Elastica Lamina Area of the Stented Carotid Artery  
Lumen CSA= Lumen area  
Lumen Diameter= Lumen diameter  
CBFV= Carotid Blood Flow Velocity of the Stented Carotid Artery

\#p NS  
*p<0.01 vs. Sedentary

*Comment to the table data:

It should pointed out that in our study, exercise significantly increased carotid blood flow velocity even after stenting further supporting an hemodynamic mechanism underlying neointimal hyperplasia reduction in exercised animals. In fact, since the stent deployment fixed the vessel area to its diameter (28 days after stent deployment, vessel remodeling is absent; indeed, there is no significant difference in between the elastic lamina area of the sedentary and swimming stented-vessels), the blood flow velocity measurements could be assumed reliable for actual blood flow volume.
Table 3. Hemodynamic Parameters in the Balloon Angioplasty Protocol.

<table>
<thead>
<tr>
<th></th>
<th>SHAM (n=8)</th>
<th>SEDEN (n=10)</th>
<th>SWIMM (n=10)</th>
<th>SED+L-NA (n=10)</th>
<th>SWIMM+L-NA (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAP (mmHg)</strong></td>
<td>128±14</td>
<td>125±12</td>
<td>93±9*</td>
<td>152±25#</td>
<td>118±18</td>
</tr>
<tr>
<td><strong>DAP (mmHg)</strong></td>
<td>84±12</td>
<td>86±10</td>
<td>65±13*</td>
<td>96±11#</td>
<td>82±11</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>322±16</td>
<td>319±17</td>
<td>240±23*</td>
<td>309±30#</td>
<td>305±29</td>
</tr>
<tr>
<td><strong>Basal Weight (gr)</strong></td>
<td>344±14</td>
<td>345±12</td>
<td>343±16</td>
<td>347±13</td>
<td>341±17</td>
</tr>
<tr>
<td><strong>Final Weight (gr)</strong></td>
<td>360±16</td>
<td>364±14</td>
<td>349±9*</td>
<td>369±18</td>
<td>348±10^</td>
</tr>
<tr>
<td><strong>Lum CSA (mm²)</strong></td>
<td>0.285±0.022‡</td>
<td>0.181±0.030</td>
<td>0.315±0.056†</td>
<td>0.158±0.027</td>
<td>0.178±0.024</td>
</tr>
<tr>
<td><strong>Lum Diameter (mm)</strong></td>
<td>0.602±0.024‡</td>
<td>0.478±0.039</td>
<td>0.632±0.056†</td>
<td>0.447±0.040</td>
<td>0.475±0.033</td>
</tr>
<tr>
<td><strong>CBFV (mm/sec)</strong></td>
<td>23.6±1.5*</td>
<td>16.9±2.0^</td>
<td>32.2±2.3*</td>
<td>11.9±1.0*</td>
<td>16.6±2.6</td>
</tr>
<tr>
<td><strong>CBF (nl/s)</strong></td>
<td>5.4±0.8*</td>
<td>2.7±0.7^</td>
<td>6.2±0.5*</td>
<td>1.8±0.3*</td>
<td>2.6±0.8</td>
</tr>
<tr>
<td><strong>WSR (sec⁻¹)</strong></td>
<td>350±10*</td>
<td>298±11^</td>
<td>519±34*</td>
<td>219±11*</td>
<td>299±19</td>
</tr>
</tbody>
</table>

**SEDEN** = Sedentary group  
**SWIMM** = Swimming group  
**SEDEN+L-NA** = Sedentary +L-NMMA group  
**SWIMM+L-NA** = Swimming+L-NMMA group  
**SAP** = Sistolic Arterial Pressure  
**DAP** = Diastolic Arterial Pressure  
**HR (bpm)** = Heart Rate (beats per minute)  
**Basal Weight** = Weight at the beginning of the study  
**Final Weight** = Weight at the end of the study  
**Neoint.** = Neointimal area at the site of Doppler measurements  
**N/M Ratio** = Neointima/media ratio at the site of Doppler measurements  
**Lum CSA** = Lumen area at the site of Doppler measurements  
**Lum Diameter** = Lumen diameter at the site of Doppler measurements  
**CBFV** = Carotid Blood Flow Velocity of the Injured Carotid Artery  
**CBF** = Carotid Blood Flow volume in the Injured Carotid Artery  
**WSR (sec⁻¹)** = Wall Shear Rate in the Injured Carotid Artery

* p<0.01 vs. All  
# p<0.01 vs. All  
^ p<0.01 vs. All except for SWIMM+L-NA  
† p<0.01 vs. All except for SHAM  
‡ p<0.01 vs. All except for SWIMM
Online Figure 2

CBFV (mm/s)

CBF (nl/s)

WSR (sec⁻¹)

SHAM  SEDEN  SWIMM  SEDEN +L-NA  SWIMM +L-NA

*  **  *