Absence of Dystrophin in Mice Reduces NO-Dependent Vascular Function and Vascular Density: Total Recovery After a Treatment with the Aminoglycoside Gentamicin

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Objectives—Mutations in the dystrophin gene causing Duchenne’s muscular dystrophy (DMD) lead to premature stop codons. In mice lacking dystrophin (mdx mice), a model for DMD, these mutations can be suppressed by aminoglycosides such as gentamicin. Dystrophin plays a role in flow (shear stress)-mediated endothelium-dependent dilation (FMD) in arteries. We investigated the effect of gentamicin on vascular contractile and dilatory functions, vascular structure, and density in mdx mice.

Methods and Results—Isolated mice carotid and mesenteric resistance arteries were mounted in arteriographs allowing continuous diameter measurements. Mdx mice showed lower nitric oxide (NO)-dependent FMD and endothelial NO synthase (eNOS) expression as well as decreased vascular density in gracilis and cardiac muscles compared with control mice. Treatment with gentamycin restored these parameters. In contrast, smooth muscle–dependent contractions as well as endothelium-dependent or -independent dilation were not affected by dystrophin deficiency or by gentamicin treatment.

Conclusion—Dystrophin deficiency induces a selective defect in flow-dependent mechanotransduction, thus attenuating FMD and eNOS expression, and may contribute to low arteriolar density. These findings open important perspectives regarding the mechanism involved in the pathophysiology of genetic diseases related to premature stop codons such as DMD. (Arterioscler Thromb Vasc Biol. 2004;24:671-676.)

Key Words: Duchenne’s muscular dystrophy ■ blood vessels ■ flow-mediated dilation ■ endothelium ■ vasodilation ■ arteriolar density

Flow (shear stress) is the main physiological stimulus that induces the release of vasoactive agents by vascular endothelial cells.1,2 Flow-mediated dilation (FMD) allows the adaptation of feeding arteries to the metabolic needs of each organ.1,2 Shear stress mechanotransduction involves extracellular matrix and cell structure proteins.2 Depolymerization of F-actin into G-actin is rapid on shear stress stimulation,3-5 and the absence of the intermediate filament vimentin markedly lowers FMD.6 Dystrophin might also play a key role in endothelial cells mechanotransduction in blood vessels, and has been widely shown to be involved in skeletal and cardiac muscle cells mechanotransduction.7-11 Nevertheless, although dystrophin is present in vascular smooth muscle cells,12-14 no obvious functional abnormality of the smooth muscle has been found in mice lacking dystrophin (mdx mice).14 On the other hand, the presence of dystrophin has recently been shown in both mouse and human endothelial cells.14 Furthermore, endothelium-dependent dilation, in response to shear flow mechanical stimulation, is markedly and selectively attenuated in mdx mice. These findings suggest a role for dystrophin in endothelial mechanotransduction of shear stress.14 This specific vascular dysfunction might disturb local blood flow supply to target organs and thus requires further investigation in patients with Duchenne’s muscular dystrophy (DMD). Indeed, ischemia has been described in skeletal and cardiac muscles in patients suffering dystrophy.15-17 Because shear stress is the main physiological stimulus triggering endothelium-dependent dilation, defects in flow-mechanotransduction might have serious short-term (acute local blood flow control) and long-term (arteries structure, protein expression) consequences. In addition, because flow regulates the expression of endothelial nitric oxide synthase (eNOS), we measured eNOS expression in arteries isolated from mdx mice. Because eNOS expression and NO production have a key role in the structural adaptation of the vessel wall to hemodynamic changes,18 we measured eNOS expression and the arteriolar density in skeletal and cardiac muscles in mdx mice.

The dystrophin mutation causes premature termination of the protein, and leads to dystrophin deficiency and impaired...
dystrophin-associated protein complex formation within the muscle cell membrane. The suppression of premature stop codons can be mediated by antibiotics such as aminoglycosides, which cause extensive misreading of the RNA and can thereby allow the formation of aberrant stop codons. Chronic treatment of mdx mice with the aminoglycoside gentamicin can suppress stop codons in the dystrophin gene and recover dystrophin expression in skeletal muscles. We tested the hypothesis that vascular mechanotransduction of shear stress could be improved in mdx mice with chronic gentamicin treatment. Indeed, restored dystrophin expression should restore the capacity of the endothelium to transduce shear stress into dilation, thus supporting the hypothesis that dystrophin has a key role in shear stress mechanotransduction in vascular endothelium. This study could also open new perspectives in the pathophysiology of genetic diseases such as DMD.

**Methods**

**Animals**

After anesthesia with pentobarbital (50 mg/kg), 12-week-old male mdx mice and their control (C57/B10; Ifla-Credo, L’Arbresle, France) were treated with gentamicin (Sigma) using a dose inducing the recovery of dystrophin in mdx mice (34 mg/kg per day for 14 days; Alzet minipumps implanted subcutaneously). Mice were then anesthetized for blood pressure measurement through a catheter placed in the left carotid artery. In another series of experiments 12-week-old male vimentin-null mice were treated with gentamicin (34 mg/kg per day for 14 days) in order to test the selectivity of the treatment. We have previously reported that FMD is markedly attenuated in vimentin-null mice. This decrease in FMD is quantitatively equivalent to that observed in mdx mice.

**Isolated Arteries**

After anesthesia with pentobarbital (50 mg/kg), the right carotid artery and mesenteric resistance arteries were isolated and cannulated at both ends in a video monitored perfusion system as described. Briefly, cannulated arteries were bathed in a physiological salt solution. Diameter changes were measured as intraluminal pressure was increased in a stepwise fashion from 10 to 150 mm Hg. Pressure was then set at 75 mm Hg and the flow was increased by steps. At the end of each experiment, arteries were bathed in a Ca2+-free physiological salt solution containing EGTA (2 mM), plus sodium nitroprusside (10 μmol/L), and pressure steps were repeated in order to determine passive arterial diameter. Contraction in response to phenylephrine, KCl (80 mM), and dilation to acetylcholine and sodium nitroprusside were tested in other arterial segments from the same mouse and mounted in an arteriograph under an intraluminal pressure of 75 mm Hg. Flow-mediated and acetylcholine-induced dilations were repeated after NO synthesis blockade with Nω-nitro-L-arginine methyl ester (L-NAME) (10 μmol/L) as described.

**Immunolocalization of Dystrophin in Isolated Arteries**

Immunostaining for dystrophin was performed as described in arteries mounted in embedding medium and frozen. Immunostaining was then performed on transverse cross section (5 μm thin) with an antidual dystrophin antibodies (anti-dys2, 1:200; Novacasta). Detection was performed with secondary antibodies conjugated to peroxidase or to biotin (and amplified by streptavidin-Texas-red). In other experiments, dystrophin immunostaining was performed on isolated mesenteric arteries cannulated in arteriographs (pressure=75 mm Hg, flow=50 μL/min). Cell membranes were first permeabilized with β-escin (90 mg/mL, 10 minutes) and antidystrophin antibodies (dys2) were then perfused for 30 minutes. A secondary antibody, bound to streptavidine and Texas-red, was used for signal amplification. Fluorescence staining was visualized using an Axioshot inverted microscope (Nikon) and an Odyssey XL confocal scanning system (Noran).

**Western-Blot of Dystrophin and NOS in Isolated Arteries**

Western-blot analysis of dystrophin was performed using antibodies directed against the carboxy terminus (dys2), the N terminus (Dys3), or the mid rod domain (dys1) of dystrophin (please see online Methods, available at http://atvb.ahajournals.org). Similar Western-blot analysis was performed with antibodies directed against the three existing forms of NOS: endothelial (eNOS), neuronal (nNOS) and the inducible (iNOS) form (please see online Methods).

**Vascular Density in Heart and Gracilis Muscles**

Sections (5 μm thin) obtained from frozen heart and gracilis muscle were incubated with anti-α-actin antibodies to identify arterioles, as described.

**Data Analysis**

Results are expressed as mean±SE. EC50 or IC50 (concentrations of agonist required to induce half maximal responses) and E_max (maximal responses) were calculated for phenylephrine, SNP, and acetylcholine in each artery. Significant differences between groups were determined by ANOVA and paired t test, or by Bonferroni’s test. Statistical significance was achieved when P<0.05.

**Results**

Body weight and blood pressure (n=10 per group) were not affected by the absence of dystrophin and/or by gentamicin treatment (Table).

**Immunolocalization and Protein Expression of Dystrophin**

Dystrophin was present in both vascular smooth muscle and endothelial cells in control and gentamicin-treated mdx mice (Figure IA and IB, available online at http://atvb.ahajournals.org), as shown using immunolabelling and confocal microscopy in cannulated arteries perfused under a pressure of 75 mm Hg. Confocal scanning had to be performed at high speed to avoid movement artifacts and shading, which somehow decreased the image sharpness. Immunolabelling and confocal microscopy analysis of dystrophin showed that the protein was present in endothelial cells, at the level of the plasma membrane, in both control and gentamicin-treated mdx mice (Figure IB). Similarly, in smooth muscle cells, dystrophin was present at the level of the plasma membrane, in control mice and in gentamicin-treated mdx mice (Figure IA). Dystrophin was not found in smooth muscle cells or endothelium cells in mdx mice mesentric (Figure IA and IB) and carotid (data not shown) arteries. Classical histo-immunology (peroxydase or texas-red staining, as described in Methods) also revealed the absence of dystrophin in arteries isolated from mdx mice and its recovery after gentamicin treatment (data not shown).

Western-blot analysis of dystrophin was performed in carotid (Figure IIA and IIB, available online at http://atvb.ahajournals.org) and mesenteric arteries (data not shown). We found that full-length dystrophin was recovered after gentamicin treatment of mdx mice with. Indeed, antibodies directed against the carboxy terminus (dys2), the N terminus (Dys3), or the mid rod domain (dys1) of dystrophin were used.
Approximately 40% of dystrophin were recovered in gentamicin-treated mdx mice (Figure IA).

### Structural Analysis of Mesenteric and Carotid Arteries

In isolated carotid and mesenteric resistance arteries bathed in a 0-calcium physiologic salt solution containing EGTA and sodium nitroprusside, a stepwise increase of intraluminal pressure induced a rise in diameter. In mdx mice, this passive diameter was similar to control mice for both carotid and mesenteric arteries (Figure II A and II B, available online at http://atvb.ahajournals.org). Passive arterial diameter was not affected by gentamicin in mesenteric and carotid arteries (Figure IIIA and IIIB). Wall thickness (Figure IIIIC and IIID) and cross sectional compliance (Figure III E and IIIF) were significantly lower in mdx than in control mice (Figure III). Wall thickness and compliance were similar in gentamycin-treated and control mice (Figure IIIC through IIIF).

### Arterial Responses to Pressure and Flow

Pressure-induced myogenic tone was not affected by dystrophin deficiency or by gentamicin treatment (Figure I A and 1 B). Myogenic tone was antagonized by FMD. Stepwise increases in flow induced significant arterial dilation (Figure 1C through 1E). In both carotid and mesenteric arteries FMD was lower in mdx than in control mice (Figure 1C and 1E). In vimentin-null mice, FMD was similar in gentamicin-treated mdx mice and control (Figure 1 C and 1 D). In vimentin-null mice,
FMD was lower than corresponding control mice, but FMD in vimentin-null mice was not improved by gentamicin treatment (Figure 1e).

NO-Dependent Dilation and NO Synthase in Mesenteric and Carotid Arteries

Inhibition of NO-synthesis decreased FMD in mesenteric (Figure 2a and 2b) and carotid arteries (data not shown). The effect of L-NAME was lower in mdx (Figure 2c) than in control mice (Figure 2d). In gentamicin-treated mice the inhibitory effect of L-NAME on FMD was similar to that in control mice (Figure 2c and 2d).

eNOS expression in carotid (Figure 3) and mesenteric arteries (data not shown) was lower in mdx than in control mice, whereas it was equivalent in gentamicin-treated mdx mice and control. nNOS expression was also decreased in mdx mice and recovered after gentamicin treatment (data not shown). The inducible form of the enzyme (iNOS) could not be detected.

Pharmacological Profile of Isolated Mesenteric and Carotid Arteries

Phenylephrine-induced contraction, as well as endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) dilation were not modified in mdx mice, relative to control mice (Table). Similarly, KCl (80 mmol/L)-induced contraction was not affected by dystrophin deficiency (Table). These parameters were not significantly affected by gentamicin in either control or mdx mice (Table).

Vascular Density in Heart and Gracilis Muscle

Arteriolar density in the right ventricle and in gracilis muscle was lower in mdx than in control mice (Figure 4). In gentamicin-treated mice, arteriolar density was similar to control mice (Figure 4).
Figure 4. Arterial density was determined in the right ventricle in control (a), mdx (b), or mdx mice treated for two weeks with gentamicin (c). Similar experiments were performed in the gracilis muscle (data not shown). Average arterial density was calculated in the 3 groups of mice (n=6 per group) in the gracilis (d) and in the cardiac muscle (e). *P<0.001; two-tail t test, vs control.

Discussion

This study demonstrates that the selective decrease in flow-mediated endothelium-dependent dilation found in mdx mice was associated with impaired NO-dependent dilation, decreased NOS expression, and lower arteriolar density. The structural and functional vascular defects found in mdx mice could be recovered after a 2-week-long treatment with the aminoglycoside gentamicin.

Although dystrophin has been shown to play a key role in force mechanotransduction in striated muscles, our recent study suggests its potential role in flow (shear stress) mechanotransduction in arteries. Endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) dilation, as well as arterial contractility, were not affected by the absence of dystrophin. Flow (shear stress) is a major stimulus for vascular cell growth, vascular remodeling, and angiogenesis. Thus, a defect in flow mechanotransduction caused by the absence of dystrophin could be deleterious and could affect blood flow supply to organs, especially when increased blood flow is required in situations such as growth. This finding is supported by our observation that arteriolar density, in both gracilis and cardiac muscles, was lower in mdx than in control mice. Indeed, ischemia occurs in skeletal and cardiac muscles of dystrophin-deficient patients, and a defect in FMD might be a possible cause of such a deficiency. Our finding showing that eNOS expression was decreased in mdx mice supports this. The low responsiveness to flow (shear stress) in mdx mice arteries might be responsible for decreased eNOS expression. Indeed, flow is a strong stimulus inducing eNOS expression, and NO-dependent and -independent production and the corresponding dilation are normal when activated by stimuli other than flow, as shown in the present study and in a previous one. Thus decreased eNOS expression is more likely the consequence of low sensitivity to flow in mdx mouse arteries. Nevertheless, the level of eNOS expression might be of importance for vascular adaptation to chronic changes in blood flow. Although microvascular dysfunction was initially suspected in DMD, no definitive evidence could be found. Decreased NO production in skeletal muscle cells through nNOS activation has been observed. Although NO is not the only vasodilator produced by microvascular endothelial cells, it remains a key factor. Indeed, we have previously shown that L-NA–resistant FMD is mediated, at least in part, by prostacyclin in mesenteric arteries. Skeletal muscle contraction induces a NOS–dependent arteriolar dilation, which is decreased in mdx mice. Similarly, disruption of the sarcoglycan-sarcospan complex and the resulting cardiomyopathy are associated with a deficiency of the coronary vasculature. In addition, the occurrence of ischemia has been shown in skeletal and cardiac muscles of dystrophin-deficient patients. These observations are in agreement with the existence of an endothelial dysfunction in the microcirculation. Nevertheless, no functional study has been performed in arteries, especially concerning endothelial function. Our actual and previous observations provide direct evidence that, in physiological conditions, arterial responses to flow are blunted in dystrophin-deficient mice. Together with previous reports showing that NO production by skeletal muscle during exercise (through the activation of the neuronal isoform of NOS) is decreased, our findings suggest a role for microvascular dysfunction in the progression of DMD via low sensitivity of arterioles to shear stress, and low eNOS expression. Consequently, arterial structural adaptation would not occur properly, resulting in insufficient vascular density.

Gentamicin treatment (2 weeks) restored FMD to control level and eNOS expression in arteries was also normalized, allowing us to postulate that blood flow supply might be normalized by gentamicin in all situations, during rest or exercise. Although 40% of the dystrophin expression was recovered after treatment, endothelial response to flow was fully recovered. Dystrophin, in mice treated with gentamicin, was a full-length protein and was detected by antibodies directed against the C terminus, the N terminus, and the mid rod domain of the protein. This study diverges from a recent work performed in 4 patients suffering from DMD. In these patients, similar treatment with gentamicin (2 weeks, same dose) recovered dystrophin expression. Nevertheless, the dose and duration of the treatment used in mice may not apply to human, as pointed out by the authors. Indeed, the choice of dose is crucial, as previously shown. Considering the seriousness of the disease and the diversity of the mutations causing DMDs and other genetic diseases with nonsense mutations, further studies in patients are needed. In this perspective, the vascular component of the disease should be
taken into account or reconsidered, in view of the novel findings provided here. In addition, other molecules related to gentamicin should be investigated in order to identify drugs with less toxicity. Gentamicin is a highly nephro- and oto-toxic drug. The recovery of normal vascular endothelial functions and structures in mesenteric and carotid arteries after only 2 weeks of treatment with gentamicin is in agreement with previous observations showing rapid (significant after 2 days) vascular remodeling due to a chronic change blood flow.18 Gentamicin treatment of mdx mice induced the recovery of NO-dependent FMD and eNOS expression. Recovery of eNOS expression may be due to normal shear stress sensing in response to dystrophin recovery in endothelial cells. In conclusion, we found that dystrophin expression can be recovered after a short treatment with the aminoglycoside gentamicin. Gentamicin allows the recovery of dystrophin by suppressing a premature stop codon in the gene encoding for dystrophin in mdx mice.19 Recovery of dystrophin expression restored flow-mediated endothelial-dependent dilation, eNOS expression, and microvascular density. These findings open important perspectives in the pathophysiology of genetic diseases related to premature stop codons as such as DMD.

Acknowledgments

This work was supported in part by a grant from the French Association against Myopathies (AFM, Association France-Myopathies), Paris, France. L.L. was a fellow of the AFM.

References

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Arterioscler Thromb Vasc Biol. 2004;24:671-676; originally published online January 29, 2004;
doi: 10.1161/01.ATV.0000118683.99628.42
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Figure Legends:

Figure I: A and B: Immunolocalization of dystrophin using immunofluorescence and confocal microscopy in mesenteric resistance arteries. Arteries were isolated from control, mdx mice or mdx mice treated for two weeks with gentamicin. Dystrophin (using Dys2 antibodies) was localized to the plasma membrane of both smooth muscle cells (left panel: A) and endothelial cells (right panel: B). This was performed in arteries perfused under a pressure of 75 mmHg and a flow of 50 µl/min. (n=6 mice per group). Due to the movement generated by the perfusion of the arteries in physiological conditions, the scanning of the vascular wall was performed at a speed of 10 images/sec.

Figure II: Expression of dystrophin in carotid arteries, using western-blot analysis. Dys1, Dys2 and Dys3 antibodies were directed against C-terminus, N-terminus and mid-rod domains of dystrophin, respectively. (n=8 mice per group).

*P <0.001; two-tail t-test, versus control.

#P <0.001; two-tail t-test, non-treated versus gentamicin-treated mdx mice.

Figure III: Passive vascular responses to pressure in carotid (left panel) and mesenteric resistance arteries (right panel) isolated from control or mdx mice treated for two weeks with gentamicin (genta, 34 mg/kg/day/14days). Arteries were submitted to stepwise increases in pressure when bathed in a Ca^{2+}-free physiological salt solution containing EGTA (2 mmol/L) and sodium nitroprusside (10 µM), thus defining the passive arterial diameter, expressed as active tone (passive diameter – active diameter) \(^6,14\) (a, b). Arterial wall thickness was
determined the same arteries (c, d). Cross-sectional compliance (e and f) was calculated from the diameter values shown in a and b. n=10 per group.

*P <0.05; two-factor ANOVA.
Fig 1

A  (smooth muscle cells)

Control

Mdx

Mdx + genta

B  (endothelial cells)

Control

Mdx

Mdx + genta
Fig. II

A

Dys-1
Dys-2
Dys-3

Cont + Genta
Mdx + Genta

B

% of control

dys-1
dys-2
dys-3

Cont
Cont + genta
Mdx
Mdx + genta
Fig. III

(a) Carotid Arteries

(b) Mesenteric Arteries

(c) Wall Thickness (µm)

(d) Wall Thickness (µm)

(e) Cross Sectional Compliance (µm²/mmHg)

(f) Cross Sectional Compliance (µm²/mmHg)
Online supplement:

Material and methods

Western-blot of dystrophin in isolated arteries.

Samples of carotid and mesenteric arteries were collected and homogenized (Ultrasonic Processor, Bioblock Scientific, France). Proteins were separated by SDS-PAGE (Mini gel protean II system, Bio-Rad, 100V, using 300 ml 25 mM Tris, 192 mM glycine, 0.1% SDS) using a 4% stacking gel followed by a 4.5% running gel. After migration, proteins were transferred (50 V, overnight, 4°C using 800 ml 25mM Tris, 192 mM glycine, 10% methanol) to PVDF blotting membranes (Immobilon-P, Millipore). Membranes were then washed in TBS-T buffer (composition: 10 mM Tris/base pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% Tween 20) and blocked for 2 hr at room temperature (5% fat free dry milk in TBS-T). Membranes incubated 90 min with the primary antibody (anti dys1, anti dys2 or anti dys3, 1:1000), washed again (3 times for 10 min) and incubated with HRP-conjugated secondary antibody (Santa Cruz, 90 min RT, 1:2000). Membranes were washed (3 times for 10 min) and Dystrophin was visualized using the ECL-Plus Chemiluminescence kit (Amersham).

Western-blot of NOS in isolated arteries.

Samples of carotid and mesenteric arteries were collected and homogenized (Ultrasonic Processor, Bioblock Scientific, France). Proteins were separated by SDS-PAGE (Mini gel protean II system, Bio-Rad, 100V, using 300 ml 25 mM Tris, 192 mM glycine, 0.1% SDS) using a 4% stacking gel followed by a 7% running gel. After migration, proteins were transferred (50 V, overnight, 4°C using 800 ml 25mM Tris, 192 mM glycine, 10% methanol) to PVDF blotting membranes (Immobilon-P, Millipore). Membranes were then washed in TBS-T buffer (composition: 10 mM...
Tris/base pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% Tween 20) and blocked for 2 hr at room temperature (5% fat free dry milk in TBS-T). Membranes incubated 2 hours with the primary antibody (anti eNOS, nNOS or iNOS 1:5000), washed again (3 times for 10 min) and incubated with HRP-conjugated secondary antibody (Amersham, 1 hours RT, 1:2000). Membranes were washed (3 times for 10 min) and eNOS was visualized using the ECL-Plus Chemiluminescence kit (Amersham).