Flow (Shear Stress)–Induced Endothelium-Dependent Dilation Is Altered in Mice Lacking the Gene Encoding for Dystrophin

Laurent Loufrani, PhD; Khalid Matrougui, PhD; Diane Gorny, BSc; Micheline Duriez; Isabelle Blanc, PhD; Bernard I. Lévy, MD, PhD; Daniel Henrion, PhD

Background—Dystrophin has a key role in striated muscle mechanotransduction of physical forces. Although cytoskeletal elements play a major role in the mechanotransduction of pressure and flow in vascular cells, the role of dystrophin in vascular function has not yet been investigated. Thus, we studied endothelial and muscular responses of arteries isolated from mice lacking dystrophin (mdx mice).

Methods and Results—Carotid and mesenteric resistance arteries 120 μm in diameter were isolated and mounted in vitro in an arteriograph to control intraluminal pressure and flow. Blood pressure was not affected by the absence of dystrophin. Pressure-induced (myogenic), phenylephrine-induced, and KCl-induced forms of tone were unchanged. Flow (shear stress)–induced dilation in arteries isolated from mdx mice was decreased by 50% to 60%, whereas dilation to acetylcholine or sodium nitroprusside was unaffected. NG-nitro-L-arginine methyl ester–sensitive flow dilation was also decreased in arteries from mdx mice. Thus, the absence of dystrophin was associated with a defect in signal transduction of shear stress. Dystrophin was present in vascular endothelial and smooth muscle cells, as shown by immunolocalization, and localized at the level of the plasma membrane, as seen by confocal microscopy of perfused isolated arteries.

Conclusions—This is the first functional study of arteries lacking the gene for dystrophin. Vascular reactivity was normal, with the exception of flow-induced dilation. Thus, dystrophin could play a specific role in shear-stress mechanotransduction in arterial endothelial cells. Organ damage in such diseases as Duchenne dystrophy might be aggravated by such a defective arterial response to flow. (Circulation. 2001;103:864-870.)

Key Words: endothelium ■ genes ■ dystrophin

Flow (shear stress)–induced dilation is a fundamental mechanism for the control of vascular tone. Shear stress is the main physiological stimulus for vascular endothelial cells, triggering the release of vasoactive agents.1–7 Its role in the control of blood flow supply to organs is fundamental.7 Flow-induced dilation allows the adaptation of feeder arteries to the metabolic needs of each organ.7,8 Mechanotransduction of shear stress involves the extracellular matrix and cell-structural proteins.8–18 Depolymerization of F-actin into G-actin is rapid with shear-stress stimulation,12,19 and the absence of the gene encoding for the intermediate filament vimentin greatly lowers the vascular response to shear stress.20 Dystrophin is a main cytoskeletal structural protein21–28 involved in skeletal and cardiac muscle cell mechanotransduction.21,28–30 Although dystrophin is present in vascular smooth muscle cells,25,31–33 no functional study in blood vessels has been performed, especially in response to mechanical stimuli such as pressure and flow, the main effectors of vascular tone and blood supply.1–8 The possibility of a specific vascular malfunction, such as a decrease in local blood flow supply to end organs, has never been investigated in such dystrophin-related diseases as Duchenne dystrophy, although it might at least accelerate damage to tissues and especially damage to cardiac and skeletal muscles. Thus, we tested the hypothesis that vascular mechanotransduction of the 2 main physical forces to which vessels are continuously subjected (pressure and flow) could involve dystrophin and that its absence might induce vascular disorders. Indeed, dystrophin has a key position between membrane structural proteins and the actin cytoskeleton, although this has never been described as precisely in vascular cells, and disruption of the actin filaments has been shown to specifically affect vascular responses to flow.12 We used carotid and mesenteric resistance arteries, which represent the 2 main types of arteries, ie, large-conductance (or compliance) arteries, whose elastic properties damp the energy produced by the ejection of blood by the heart at each systole, and resistance arteries, whose muscular tone and endothelial relaxing capacity regulate blood flow supply to organs.
Methods

Isolated Arteries
Mdx mice and their controls (C57-B10) were obtained from Iffa-Credo (L’Arbresle, France). They were anesthetized for blood pressure measurement through a catheter in the left carotid artery. Then right carotid and mesenteric arteries were isolated and cannulated at both ends in a video-monitored perfusion system (LSI) as previously described. Briefly, arteries were bathed in a physiological salt solution (pH 7.4, PO₂ 160 mm Hg, PCO₂ 37 mm Hg). Pressure was controlled by a servoperfusion system, and flow was generated by a peristaltic pump. Diameter changes were measured with intraluminal pressure was increased from 10 to 125 mm Hg. We then set pressure at 75 mm Hg and increased flow by steps. At the end of each experiment, arteries were perfused and superfused with a calcium-free physiological salt solution containing EGTA (10 mmol/L, and sodium nitroprusside (10 μmol/L), and pressure steps were repeated to determine the passive diameter of the arteries (contractions to phenylephrine (1 nmol/L to 10 μmol/L), KCl (80 mmol/L), and calcium (0.1 to 1 mmol/L in a calcium-free medium + 80 mmol/L KCl) were tested separately. Dilations to acetylcholine and sodium nitroprusside were tested after preconstriction of the arteries with phenylephrine (50% of the maximal contraction).

Histomorphometric Analysis
Histomorphometry of the arteries was performed as previously described on segments of arteries previously mounted in the arteriograph as described above. Pressure was set at 75 mm Hg, and vessels were fixed in 10% formaldehyde in saline solution for 30 minutes and sectioned at 10 μm thick. Morphometric analysis was performed with an automated image processor.

Immunolocalization of Dystrophin and In Situ Confocal Microscopy
Segments of arteries were mounted in embedding medium (Miles, Inc) and frozen in isopentane. Immuno staining was then performed on transverse cross sections 5 μm thick incubated overnight at 4°C with anti-dystrophin antibodies (anti-dys2, 1:20, Novacastra) and then incubated for 30 minutes at 37°C with anti-rabbit antibodies conjugated to peroxidase (Amersham). Samples were mesenteric resistance or carotid arteries, gracilis muscle, and heart from mdx and control mice, as well as human internal mammary and mesenteric arteries. Positive staining was visualized as a brown-orange staining by video microscopy.

In another group of experiments, immunostaining of dystrophin was performed in isolated mesenteric arteries from control and mdx mice mounted in an arteriograph under a pressure of 75 mm Hg and a flow of 50 μL/min, so that vascular cells were left in physiological condition. Cell membranes were permeabilized with β-escin (90 mg/mL, 10 minutes) to allow antibodies to reach dystrophin. A secondary antibody (anti-IgG) bound to streptavidin and Texas Red was used to labeled anti-dystrophin antibodies. Fluorescence staining was visualized with an Axiohot inverted microscope (Nikon) equipped with an Odyssey XL confocal scanning system (Noran Instruments), which allowed us to visualize staining of endothelial cells in the luminal side of the perfused artery.

Finally, we also used human mammary and epiploic arteries to immunolocalize dystrophin in endothelial and smooth muscle cells, as described above. These human arteries were isolated from excess material normally discarded after surgery.

Statistical Analysis
Results were expressed as mean±SEM. EC₅₀ or IC₅₀ (concentration of agonist required to induce half the maximum response) and E₅₀ (maximal response) were calculated for each artery. Significance of the differences between groups was determined by 1- or 2-factor ANOVA or ANOVA for consecutive measurements, when appropriate. Means were compared by paired t test or by Bonferroni’s test for multigroup comparisons. Values of P<0.05 were considered to be significant.

Results

Animals
Body weight was not affected by the absence of dystrophin (33±3 versus 35±3 g, mdx versus control mice, n=12 per group). Similarly, blood pressure was normal in mdx mice (mean arterial pressure, 86±5 mm Hg in mdx versus 88±6 mm Hg in control mice, n=12 per group).

Figure 1. Vascular response to flow. Typical recordings showing changes in diameter in response to step increases in flow in mesenteric resistance arteries isolated from control (a) or mdx (b) mice and mounted in an arteriograph, under a pressure (P, top recordings) of 75 mm Hg. Flow-induced dilation obtained in mesenteric (c) and carotid (d) arteries was strongly attenuated in mdx mice. n=14 per group. *P<0.001; 2-factor ANOVA, control vs mdx.

Figure 2. Changes in diameter in response to step increases in pressure in mesenteric resistance (myogenic tone, top) and carotid (basal tone, bottom) arteries isolated from mdx and control mice. n=14 per group. No significant difference; 2-factor ANOVA, control vs mdx.
Isolated Arteries

In isolated carotid and mesenteric resistance arteries under a physiological level of intraluminal pressure, a basal (myogenic) tone develops, which was antagonized by flow (shear stress)–induced dilation. Thus, increasing flow by steps induced a progressive dilation (Figure 1). In both carotid and mesenteric resistance arteries, flow (shear stress)–induced dilation was strongly attenuated in mdx mice (Figure 1). Pressure (tensile stress)–induced tone (myogenic in resistance arteries) was unaffected by the absence of dystrophin (mdx mice) in both types of vessels (Figure 2). Other endothelium-dependent (acetylcholine) or -independent (sodium nitroprusside) forms of dilation were not modified in mdx mice in either carotid or resistance arteries (Table). Similarly, contractions to calcium, KCl, or phenylephrine (Table), in addition to basal tone due to pressure (Figure 2), were not affected by the lack of dystrophin.

Pharmacological Profile of Mouse Arteries

<table>
<thead>
<tr>
<th></th>
<th>mdx</th>
<th>Control</th>
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<tbody>
<tr>
<td>Mesenteric arteries</td>
<td></td>
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<tr>
<td>SNP</td>
<td></td>
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<tr>
<td>IC_{50}, mmol/L</td>
<td>43±7</td>
<td>32±8</td>
</tr>
<tr>
<td>Imax, %</td>
<td>100±1</td>
<td>100±1</td>
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<tr>
<td>ACh</td>
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<tr>
<td>IC_{50}, mmol/L</td>
<td>78±9</td>
<td>87±8</td>
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<tr>
<td>Imax, %</td>
<td>99±2</td>
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<tr>
<td>PE</td>
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<tr>
<td>EC_{50}, mmol/L</td>
<td>28±4</td>
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</tr>
<tr>
<td>E_{max}, μm</td>
<td>86±8</td>
<td>93±8</td>
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<tr>
<td>Ca^{2+}</td>
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<tr>
<td>EC_{50}, mmol/L</td>
<td>0.2±0.04</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>E_{max}, μm</td>
<td>105±11</td>
<td>128±20</td>
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<tr>
<td>Carotid arteries</td>
<td></td>
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</tr>
<tr>
<td>SNP</td>
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<tr>
<td>IC_{50}, mmol/L</td>
<td>61±17</td>
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<tr>
<td>Imax, %</td>
<td>78±5</td>
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<tr>
<td>ACh</td>
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<td>Imax, %</td>
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<tr>
<td>PE</td>
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<td>EC_{50}, mmol/L</td>
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<tr>
<td>E_{max}, μm</td>
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<tr>
<td>Ca^{2+}</td>
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<tr>
<td>EC_{50}, mmol/L</td>
<td>0.34±0.06</td>
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<td>E_{max}, μm</td>
<td>74±7</td>
<td>72±6</td>
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<tr>
<td>Contraction to KCl</td>
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<td>Carotid arteries, μm</td>
<td>93±8</td>
<td>100±12</td>
</tr>
<tr>
<td>Mesenteric arteries, μm</td>
<td>112±8</td>
<td>118±6</td>
</tr>
</tbody>
</table>

EC_{50} and IC_{50} indicate concentration necessary to reach 50% of the maximal effect; E_{max} and Imax, maximal effect of the drug (n=8 per group). Contraction to phenylephrine (PE), KCl (80 mmol/L, and calcium (Ca^{2+}) and dilation to acetylcholine (ACh) and sodium nitroprusside (SNP) were obtained in mesenteric resistance arteries and carotid arteries isolated from mice lacking the gene for dystrophin (mdx) and their control.

No significant difference between mdx and control mice was found.

Isolated Arteries

In isolated carotid and mesenteric resistance arteries under a physiological level of intraluminal pressure, a basal (myogenic) tone develops, which was antagonized by flow (shear stress)–induced dilation. Thus, increasing flow by steps induced a progressive dilation (Figure 1). In both carotid and mesenteric resistance arteries, flow (shear stress)–induced dilation was strongly attenuated in mdx mice (Figure 1). Pressure (tensile stress)–induced tone (myogenic in resistance arteries) was unaffected by the absence of dystrophin (mdx mice) in both types of vessels (Figure 2). Other endothelium-dependent (acetylcholine) or -independent (sodium nitroprusside) forms of dilation were not modified in mdx mice in either carotid or resistance arteries (Table). Similarly, contractions to calcium, KCl, or phenylephrine (Table), in addition to basal tone due to pressure (Figure 2), were not affected by the lack of dystrophin.

Blockade of NO synthesis by NG-nitro-L-arginine methyl ester (L-NAME) reduced flow-induced dilation in both types of arteries (Figure 3, top). L-NAME was less efficient in arteries from mdx mice stimulated by flow than in control mice (Figure 3, bottom). Direct stimulation of cGMP-dependent dilation (endothelium-independent) with sodium nitroprusside was unaffected in mdx mice (Table).

Figure 4. Arterial wall thickness (top) and wall-to-lumen ratio (bottom) in mesenteric resistance and carotid arteries isolated from mdx and control mice (n=6 to 8 per group). *P<0.01; 2-factor ANOVA, control vs mdx.
Inhibition of angiotensin II or endothelin-1 receptors did not affect flow-induced dilation in arteries from mdx mice (n=6 per group, data not shown).

Histomorphometry and Passive Properties of the Vascular Wall

Although no significant change in arterial wall thickness (Figure 4) or passive diameter (Figure 5, mesenteric arteries, and Figure 6, carotid arteries) was found, arterial wall structure was affected by the absence of dystrophin, as visualized by a larger wall-to-lumen ratio (Figure 4) and a lower compliance and distensibility of the carotid artery (Figure 6).

Immunolocalization of Dystrophin

The protein dystrophin was present in both vascular smooth muscle cells and endothelial cells in control mice (absent in mdx mice) but also in human internal mammary and mesenteric resistance arteries (Figure 7). Confocal scanning of isolated arteries mounted in an arteriograph to maintain physiological levels of pressure and flow in the lumen of the arteries shows that dystrophin is present in both endothelial and smooth muscle cells. In these cells, dystrophin was located at the level of the plasma membrane (Figure 8).

Discussion

This is the first study of vascular function in relation to the genetic deficiency in dystrophin. Interestingly, in mice lacking the gene for dystrophin, vascular reactivity (endothelial and muscular) was normal, with the exception of flow (shear stress)–induced dilation, which was strongly attenuated.

Although dystrophin has been clearly shown to play a key role in force mechanotransduction in striated muscles, its possible role in the mechanotransduction of pressure and flow has never been investigated. Flow and pressure are 2 of the main factors involved in the control of blood vessel tone and blood flow supply, and understanding of their transduction pathway(s) is fundamental. Surprisingly, in both isolated carotid and mesenteric resistance arteries, pressure (tensile stress)–induced tone (myogenic in resistance arteries) was unaffected by the absence of dystrophin, whereas flow (shear stress)–induced dilation was strongly attenuated in mdx mice. Thus, only mechanotransduction of shear stress at the surface of endothelial cells, not that to pressure exerted on the whole vessel wall, was attenuated. Furthermore, in this mouse...
model with a strong attenuation of flow-induced dilation, blood pressure was normal. This and our previous observations in mice lacking the gene encoding for vimentin and in rats rendered hypertensive with a chronic infusion of endothelin strengthen the hypothesis that flow dilation has a key role in the control of local blood flow but is not necessarily and/or directly related to the basal level of systemic blood pressure.

Flow dilation was specifically attenuated in mdx mice. Other endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) dilations were not modified in mdx mice. Similarly, contractions to calcium, KCl, or phenylephrine, in addition to myogenic tone due to pressure, were not affected by the lack of dystrophin, showing that no endothelial dysfunction and no defect in smooth muscle contractility or vasorelaxant properties could be involved in the reduction in dilation to shear stress found in arteries from mdx mice.

Although no significant change in arterial wall thickness or passive diameter was found, arterial wall structure was affected by the absence of dystrophin, as visualized by a larger wall-to-lumen ratio and a lesser compliance and distensibility (Figure 2). Nevertheless, these changes cannot explain a change only in endothelial response to flow, with no effect on other forms of tone. Indeed, in both mdx and control mice, arterial tone before induction of flow dilation was similar.

Nitric oxide (NO) is the major relaxing agent released by the endothelium after flow stimulation and the blockade of its synthesis was less efficient in arteries from mdx mice.
mice stimulated by flow, whereas direct stimulation of cGMP-dependent dilation with sodium nitroprusside was unaffected in mdx mice. Thus, arteries from mdx mice are less able to produce NO in response to shear stress. In addition, arteries from mdx mice did not produce more endothelium-derived vasoconstrictor agents when stimulated by flow, because angiotensin II or endothelin-1 receptor inhibition did not affect flow-induced dilation in arteries from mdx mice. Thus, the lack of dystrophin caused a specific defect in the transduction of shear stress into a dilation through the capability of the NO-cGMP pathway in endothelial cells to dilate normally to other relaxing stimuli. This attenuation in flow-induced dilation might lead to a lesser adaptation to increases in blood flow in organs when a metabolic need requires a higher blood flow supply. In addition, because flow (shear stress at the surface of the endothelial cells) is a major stimulus for vascular cell growth and angiogenesis,\(^4\) \(^8\) \(^42\) \(^43\) \(^45\) a defect in flow-mechanotransduction due to the absence of dystrophin could be deleterious for the angiogenic process, and consequently, blood flow supply to organs might be affected when an increase in blood flow is required, as, for instance, in exercise. In support of this statement, skeletal muscle contraction induces a neuronal NO synthase-dependent arteriolar dilation, which is decreased in mdx mice. This lower dilation has been attributed to a lower capacity of the skeletal muscle to produce NO,\(^46\) but in view of the present study, we can also postulate that the increase in blood flow required for the contraction might not be high enough in mdx mice, leading to a lesser NO production in blood vessels as well. Also in support of our hypothesis, the occurrence of ischemia has been shown in skeletal and cardiac muscles of dystrophin-deficient patients.\(^47\)\(^48\)

Finally, the protein dystrophin was present in both vascular smooth muscle cells and endothelial cells in control mice (absent in mdx mice) and also in human internal mammary and mesenteric resistance arteries. This location is in agreement with the studies performed in skeletal and cardiac muscle cells\(^39\) and strengthens the possibility that dystrophin in vascular endothelial cells plays a major role in mechanotransduction. Flow-mechanotransduction also involves integrins.\(^49\) Although it is tempting to link the 2 proteins in the same pathway, such a possibility requires further investigation. In addition, blockade of integrins with RGD peptides may suppress totally flow-induced dilation,\(^49\) whereas the absence of dystrophin in mdx mice decreased the response to 40% to 50% of that in control mice. This could reflect an adaptation of the endothelial cells to the chronic absence of dystrophin, and other proteins, such as dystrophin-related proteins, could be involved in flow-mechanotransduction in mdx mice. Finally, the transduction pathway beyond dystrophin leading to the activation of NO synthesis, and especially the type of kinases involved, also remains to be elucidated.

In conclusion, we found that dystrophin plays a key role in the mechanotransduction of shear stress by the vascular endothelium in both large and resistance arteries. The present findings support the concept that some elements of the cytoskeleton, with a central role for dystrophin, may specifically transduce the signal from shear stress to the enzymatic dilator machinery in vascular endothelial cells. This observation might be of importance to better understand the development and possibly to improve the treatment of dystrophin-related diseases.

### Acknowledgments

This study was supported in part by a grant from the French Association Against Myopathies (AFM), Paris, France. Dr Loufrani is a fellow of the French Association Against Myopathies (AFM), Paris, France.

### References

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_Circulation_. 2001;103:864-870
doi: 10.1161/01.CIR.103.6.864

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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