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Nitric oxide donor molsidomine favors features of atherosclerotic plaque stability and reduces myocardial infarction in mice

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1 Equal contribution.

ABSTRACT

Nitric oxide (NO) donors are commonly used for the prevention and treatment of ischemic heart disease. Besides their effects on the heart, NO donors may also prevent hypoxic brain damage and exert beneficial effects on atherosclerosis by favoring features of plaque stability. We recently described that apolipoprotein E (ApoE) deficient mice with a mutation in the fibrillin-1 (Fbn1) gene (ApoE⁻/⁻ Fbn1C1039G⁺/⁻) develop accelerated atherosclerosis, plaque rupture, myocardial infarction, cerebral hypoxia and sudden death. In the present study, we evaluated the effects of chronic treatment with the NO donor molsidomine on atherosclerotic plaque stability, cardiac function, neurological symptoms and survival in the ApoE⁻/⁻ Fbn1C1039G⁺/⁻ mouse model. Female ApoE⁻/⁻ Fbn1C1039G⁺/⁻ mice were fed a Western diet (WD). After 8 weeks of WD, the mice were divided into two groups receiving either molsidomine via the drinking water (1 mg/kg/day; n = 34) or tap water (control; n = 36) until 25 weeks of WD. Survival tended to increase after molsidomine treatment (68% vs. 58% in controls). Importantly, atherosclerotic plaques of molsidomine-treated mice had a thicker fibrous cap (11.1 ± 1.2 vs. 8.1 ± 0.7μm) and showed an increased occurrence of plaque macrocalcifications (30% vs. 0%), indicative of a more stable phenotype. Molsidomine also improved cardiac function, as fractional shortening was increased (40 ± 2% vs. 27 ± 2%) combined with a decreased end diastolic (3.1 ± 0.2 vs. 3.9 ± 0.2 mm) and end systolic diameter (1.9 ± 0.1 vs. 2.9 ± 0.2 mm). Furthermore, perivascular fibrosis (23 ± 2 vs. 30 ± 2%) and the occurrence of myocardial infarctions (12% vs. 36%) was significantly reduced. Track width, a measure of the animal’s hind limb base of support and representative of hypoxic brain damage, was also normalized as a result of molsidomine treatment (2.54 ± 0.04 vs. 2.91 ± 0.09 cm in controls). These findings demonstrate that the NO donor molsidomine improves cardiac function, reduces neurological symptoms and enhances atherosclerotic plaque stability.

1. Introduction

Nitric oxide (NO) is involved in a wide range of physiological processes in the cardiovascular system [1]. In blood vessels, NO is an important determinant of vascular tone, regulating blood pressure and vascular compliance. Moreover, NO maintains vascular integrity by inhibiting smooth muscle cell (SMC) proliferation, platelet aggregation and leukocyte adhesion to the endothelium [1–3]. In the heart, NO plays a major role in determining the resting vascular tone of coronary resistance vessels [4,5] and is produced in cardiac SMC, where it regulates myocardial contractility [2,5]. Physical or biological injury to the endothelium impairs the production and/or bioavailability of NO, which may play a role in hypertension, repertfusion injury, atherosclerosis and myocardial depression [1,6,7].

In line with these findings, patients suffering from ischemic cardiac failure and atherosclerosis might benefit from chronic treatment with an exogenous NO donor. The NO donor molsidomine has the advantage that it releases NO non-enzymatically, avoiding tolerance. Our group and others have previously shown that molsidomine, which exerts its effects through the metabolite 3-morpholinosydnonimine (SIN-1), favoured cardiac function [8–12], decreased platelet aggregation [11,13,14] and signs of oxidative stress and increased features of plaque stability [15] in humans, as well as in animal models. Moreover, patients treated with molsidomine for over a year had significantly lower plasma levels of soluble adhesion molecule ICAM-1, which may slow down the progression of atherosclerosis [16]. However, the beneficial effects of molsidomine are dose-dependent, since high amounts can generate peroxynitrite, causing enhanced oxidative stress, thereby aggravating atherosclerosis [17].

We recently described a novel atherosclerosis model, apolipoprotein E-deficient mice with a mutation in the fibrillin-1 gene (ApoE⁻/⁻ Fbn1C1039G⁺/⁻), characterized by accelerated atherosclerosis, plaque...
rupture, myocardial infarction, cerebral hypoxia and sudden death [18]. In these mice, the elastic fibers in the vessel wall are fragmented, resulting in enhanced arterial stiffness and thereby mimicking vascular aging [19]. As a result, ApoE<sup>-/-</sup>Fbn1<sup>C1039G +/+</sup> mice develop large and highly unstable plaques with a phenotype similar to human plaques. Moreover, these mice develop coronary artery plaques and left ventricular hypertrophy, resulting in decreased cardiac function and myocardial infarction. In the present study, we evaluated the effects of chronic molsidomine treatment in ApoE<sup>-/-</sup>Fbn1<sup>C1039G +/+</sup> mice on atherosclerotic plaque stability, cardiac function, neurological symptoms and sudden death.

2. Methods

2.1. Mice

Female ApoE<sup>-/-</sup>Fbn1<sup>C1039G +/+</sup> mice (n = 70, 6 weeks of age) were fed a Western-type diet (WD; TD88137, Harlan Teklad, Madison, WI) for up to 25 weeks. After 8 weeks of WD feeding, animals were divided into two groups receiving either molsidomine via the drinking water (1 mg/kg body weight/day; n = 34) or tap water (control; n = 36) until 25 weeks of WD. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle with free access to water and food. Mice were inspected daily for occurrence of sudden death. At the end of the experiment, mice were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and blood samples were taken from the retro-orbital plexus for plasma cholesterol analysis. Subsequently, animals were euthanized with an overdose of sodium pentobarbital (250 mg/kg, i.p.). Total plasma cholesterol was analysed by means of a commercial kit (Randox, Crumlin, UK). All experiments were approved by the ethical committee of the University of Antwerp and were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2. Echocardiography

Trans-thoracic echocardiograms (ECGs) of ApoE<sup>-/-</sup>Fbn1<sup>C1039G +/+</sup> mice were performed at the start (baseline, 8 weeks on WD) and after 12 weeks of molsidomine treatment. ECGs were performed on anesthetized mice (isoflurane, IsoFlo, Abbott) using a Toshiba diagnostic ultrasound system (SSA-700A), equipped with a 15 MHz transducer.

2.3. Blood pressure

Peripheral blood pressure was measured with a tail cuff (Coda<sup>™</sup> High throughput, Kent Scientific, Torrington, CT). Mice were trained for four weeks before the baseline measurement and every week thereafter, until 12 weeks of molsidomine treatment, when a final measurement was performed.

2.4. Motor coordination

Track width was analysed after 12 weeks of treatment as described previously [20]. Briefly, ink was applied to the animals’ hind paws and the mice were required to walk on a strip of paper towards a dark goal box. The median value of a minimum of 10 measurements per mouse was used.

2.5. Histological evaluation

After euthanasia or sudden death, mice were dissected and aorta, heart, spleen and kidneys were weighed (dry weight). Heart and arteries were post-fixed in 4% formaldehyde (pH 7.4) for 24 h, processed and embedded in paraffin. Histological analyses were performed on serial cross sections (5 μm) of the brachiocephalic artery and proximal ascending aorta. The percentage stenosis and the size of atherosclerotic plaques and necrotic cores was measured on hematoxylin and eosin (H&E) stained sections. The necrotic cores were defined as acellular/anuclear areas (negative for hematoxylin) with a 3000 μm<sup>2</sup> threshold implemented to avoid counting of very small H&E negative areas that likely do not represent areas of necrosis. The presence of macrocalcifications, identified as basophilic (dark purple) crystalline structures, was determined on H&E stained sections. The presence of 3-nitrotyrosine in plaques of the proximal ascending aorta was identified by immunohistochemical staining with an anti-nitrotyrosine antibody (sc-55,256, Santa cruz). Cellular composition of the atherosclerotic plaques was analysed by immunohistochemistry using anti-manose receptor (macrophages, ab64693, Abcam) and anti-α-smooth muscle cell actin (F3777, Sigma-Aldrich). Fibrous cap thickness was determined as the median value of 10 measurements per atherosclerotic plaque on α-SMC actin (F3777, Sigma-Aldrich) stained sections. Collagen content was determined on Sirius red stained sections with light microscopy (total collagen) and polarized light (type I collagen). Fibrous cap thickness was determined as the median value of 10 measurements per atherosclerotic plaque on α-SMC actin (F3777, Sigma-Aldrich) stained sections. The ICAM-1 expression of the endothelial cells was measured via immunohistochemical staining with an anti-vWF (PC054, The Binding Site) and anti-ICAM-1 antibody (550287, BD Biosciences). The length of CD31 positive cells along the luminal border was quantified in a complete cross-section of the proximal ascending aorta and ICAM-1 positivity was expressed as percentage of CD31 positivity. Intraplaque neovascularization and hemorrhages in the left common carotid artery were examined on slides that were stained with anti-vWF (PC054, The Binding Site) and anti-TER119 (550,565, BD Biosciences), respectively. The occurrence of myocardial infarctions, coronary plaques and perivascular fibrosis, measured as the perivascular collagen area divided by the luminal area (PVCA/LA) of 10 coronary arteries per mouse, was analysed on Mason's trichrome stained transversal sections (cut from the middle of the heart to the apex). The myocardial infarctions were defined as large fibrotic areas with infiltration of inflammatory cells. If plaques were present in the coronary arteries, the plaque size and percentage of stenosis were measured on Mason's trichrome stained sections. All images were acquired with the Universal Grab 6.1. (IDL software, Exelix, Boulder, CO) using an Olympus BX40 microscope (Tokyo, Japan). Images were quantified using Image J software (NIH).

2.6. Statistical analyses

Normally distributed data are expressed as mean ± SEM and non-normally distributed variables are represented as median [min-max]. Statistical analyses were performed using SPSS software (version 24, SPSS Inc., Chicago). Statistical tests are specified in the figure and table legends. Histological data of the proximal ascending aorta and left common carotid artery only include mice that survived the experiment. Data on MI and coronary arteries include all mice. Differences were considered significant at P < 0.05.

3. Results

Body weight and total plasma cholesterol was similar in treated and control mice (Table 1). Survival improved slightly, albeit not significantly, after molsidomine treatment (68% vs. 58% in controls, Table 1). A marked decrease in kidney and spleen weight was observed in the molsidomine-treated group, whereas lung weight remained equal between both groups (Table 1).

3.1. Molsidomine improves atherosclerotic plaque stability

Molsidomine did not affect plaque size in the proximal ascending aorta nor the degree of stenosis in ApoE<sup>-/-</sup>Fbn1<sup>C1039G +/+</sup> mice (Table 2, Fig. S1). Furthermore, the NO donor had no effect on the size of the necrotic core, the amount of macrophages, smooth muscle cells (SMCs),
Atherosclerotic plaque characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control Molsidomine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel area (×10³ µm²)</td>
<td>1379 ± 100</td>
</tr>
<tr>
<td></td>
<td>1284 ± 117</td>
</tr>
<tr>
<td>Plaque thickness (×10³ µm²)</td>
<td>798 ± 67</td>
</tr>
<tr>
<td></td>
<td>792 ± 73</td>
</tr>
<tr>
<td>Stenosis (%)</td>
<td>58 ± 3</td>
</tr>
<tr>
<td></td>
<td>62 ± 2</td>
</tr>
<tr>
<td>Necrotic core (%)</td>
<td>8.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>10.1 ± 1.4</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>Total collagen (%)</td>
<td>39.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>39.7 ± 1.2</td>
</tr>
<tr>
<td>Type 1 collagen (%)</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Smooth muscle cells (%)</td>
<td>15.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>14.2 ± 0.9</td>
</tr>
<tr>
<td>Fibrous cap thickness (µm)</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td>ICAM-1 positive ECs (%)</td>
<td>94.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>94.8 ± 1.4</td>
</tr>
</tbody>
</table>

Survival was evaluated using a Pearson’s chi-square test. Body weight, plasma cholesterol and organ weights are presented as mean ± SEM (n = 13–18 per group) and analysed by an independent samples t-test. *P < 0.05, **P < 0.01.

3.3. Molsidomine improves motor function of ApoE⁻/⁻Fbn1C1039G+/- mice

Track width measurements were previously validated as a reliable marker for hypoxic brain damage in ApoE⁻/⁻Fbn1C1039G+/- mice [20]. Molsidomine was able to inhibit the increase in track width normally observed in ApoE⁻/⁻Fbn1C1039G+/- mice fed a WD. Both the median and maximum track width values were significantly lower after 12 weeks of molsidomine treatment compared with control mice (Fig. 3A–B). Track width was also positively correlated with plaque size in the proximal ascending aorta (Fig. 3C).

4. Discussion

The present study confirms the beneficial effects of molsidomine in the treatment of ischemic and left ventricular cardiac failure, as seen in humans [8]. Moreover, molsidomine prevented a loss in motor function and favoured atherosclerotic plaque stability. Molsidomine is a veno-dilator that reduces the cardiac preload, thereby lowering cardiac output, ventricular work and myocardial oxygen consumption [9]. Furthermore, it evokes a local dilatation of the coronary arteries, reducing coronary artery stenosis and preventing cardiac ischemia [10]. ApoE⁻/⁻Fbn1C1039G+/- mice are characterized by the development of pronounced atherosclerosis, which is caused by stiffening and inflammation of the arteries, as a result of elastin fragmentation in the vessel wall [18,19]. These mice develop progressive cardiac failure, coronary artery plaque, myocardial infarctions and hypoxic brain damage, similar as seen in humans [18]. Therefore, they could benefit from treatment with an exogenous NO donor. We chose for a chronic treatment with a low dose of molsidomine (1 mg/kg/day). It is very important to select the most optimal dose, because high dosages can generate peroxynitrite, causing enhanced oxidative stress, which may lead to endothelial dysfunction [17]. This was also reported during long-term treatment with other NO donors (nitroglycerin, isosorbide mononitrate) [21]. Therefore, it is important to evaluate 3-nitrotyrosine as a marker of peroxynitrite formation. In the current study, we could not detect a difference between 3-nitrotyrosine in plaques of control and molsidomine-treated mice. Thus, molsidomine at a dose of 1 mg/kg/day does not lead to oxidative stress-related side effects [15]. Importantly, molsidomine significantly improved cardiac function.
mean ± SEM and were analysed by an Independent samples
MI) and coronary stenosis (in mice with coronary plaque) are presented as
were evaluated using a Pearson's chi-square test. Infarcted area (in mice with
ESD) in increased FS and left ventricular hypertrophy (decreased EDD and
Blood pressure is presented as mean ± SEM (n=13–18 per group) and ana-
Table 3
Blood pressure, MI and coronary artery plaque characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Molsidomine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP</td>
<td>112 ± 7</td>
<td>111 ± 5</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>79 ± 7</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>35 ± 1</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Myocardial infarction (MI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence of MI</td>
<td>12/33 (36%)</td>
<td>4/33 (12%)</td>
</tr>
<tr>
<td>Infarcted area (%)</td>
<td>1.8 ± 0.4</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Coronary plaque</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occurrence of coronary plaque</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stenosis (%)</td>
<td>58 ± 9</td>
<td>72 ± 8</td>
</tr>
</tbody>
</table>

Blood pressure is presented as mean ± SEM (n=13–18 per group) and analysed by Independent samples t-test. Occurrence of MI and coronary plaque were evaluated using a Pearson's chi-square test. Infarcted area (in mice with MI) and coronary stenosis (in mice with coronary plaque) are presented as mean ± SEM and were analysed by Independent samples t-test. * P < 0.05 vs. control mice.

Therefore NO donors have been investigated as a therapeutic strategy for preventing hypoxic brain damage. For instance, treatment with NO donors prevented hypoxia-ischemia induced neurological damage in rats [23,24] and a reduced brain infarct size was observed in several animal models of stroke [25]. The trials involving the use of molsidomine are limited, but a recent observational study showed that after subarachnoid haemorrhage, molsidomine treatment resulted in less vasospasm-related infarctions and a better clinical outcome [26]. These beneficial effects are not only attributed to the improved cerebral blood flow, but also to other cellular and metabolic effects, such as the inhibition of neuronal apoptosis after ischemia [26]. Thus, the current study further supports the finding that molsidomine treatment can prevent hypoxic brain damage, but further clinical trials are needed to confirm these results.

We also investigated whether molsidomine could have beneficial effects on atherosclerosis. We did not observe a reduction in plaque size or degree of stenosis, confirming our previous observations [15]. Although macrophage, SMC and total collagen content remained unaltered, the NO donor significantly increased fibrous cap thickness, a major determinant of tensile strength of the cap. NO is able to down-regulate p53 and reduce the expression of pro-inflammatory cytokines IL-1 and TNF-α, thereby decreasing apoptosis and inflammation [27]. In this way, SMC survival is favoured, leading to a thicker fibrous cap and a more stable plaque phenotype. Moreover, molsidomine treatment resulted in a significant increase in plaque macrocalcifications located near the medial border. It is known that calcified coronary nodules are a feature of acute coronary syndromes [28,29]. However, the type of calcification observed in the present study does not resemble calcified nodules since it is not eruptive and does not protrude into the lumen.

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Cardiac parameters of control and molsidomine-treated mice. (A–C) ApoE−/−/Fbn1C1039G+/− mice treated with molsidomine (n=9) showed a significantly lower end systolic diameter (ESD) and end diastolic diameter (EDD) as compared to controls (n=9). Moreover, fractional shortening (FS) was significantly higher in the molsidomine treatment group. Independent samples t-test; **P < 0.01 and ***P < 0.001. (D) Molsidomine treatment normalized heart weight of ApoE−/−/Fbn1C1039G+/− mice (n=15 in both groups). Independent samples t-test; *P < 0.05. (E) Histological images of Masson's trichrome stained hearts showing a myocardial infarction (blue staining, arrow) in the right ventricle of the control-treated mouse. Scale bar=500μm. (F) Masson's trichrome staining of coronary arteries indicated that perivascular fibrosis, measured as perivascular collagen area (PVCA) divided by the lumen area (LA), was significantly reduced in molsidomine-treated mice as compared to controls. 2-way ANOVA; **P < 0.01. Scale bar = 100μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
circumstances, remnants of apoptotic SMCs, which remain in the plaque as matrix vesicles, will act as nucleating structures for calcification [38,39]. These microcalcifications are associated with enhanced plaque progression [32] and have the potential to increase the occurrence of plaque rupture via the induction of biomechanical stress [32,40,41]. Considering the latter, the location in the plaque is of great importance, because only microcalcifications in the fibrous cap negatively affect the mechanical stability, while calcifications found in the necrotic core or at the medial border, as seen in the present study, do not contribute to plaque instability [32,41]. In contrast to microcalcifications, macrocalcifications are considered plaque stabilizing, because they are the result of a healing process that is able to separate the inflammatory plaque content from the vessel lumen [42]. These large calcified areas are formed when the initial inflammation in the plaque is resolved, leading to a tissue repair response and osteogenesis. Therefore, large plaque calcifications have been associated with decreased fibrous cap inflammation [43]. As bone formation is inhibited by chronic inflammation, microcalcifications will not evolve into macrocalcifications, unless the inflammatory status of the plaque decreases [37]. NO has anti-inflammatory properties, which can explain the increase in plaque macrocalcifications [27]. Molsidomine treatment was introduced at a timepoint where advanced atherosclerotic plaques are present in ApoE<sup>−/−</sup> Fbn1<sup>C1039G−/+</sup> mice, which is the ideal environment for the development of microcalcifications, but the anti-inflammatory effects of the therapy allowed osteogenesis to occur followed by the formation of macrocalcifications. Moreover, we could detect a clear increase in collagen type I in the calcified plaques, which is also a typical hallmark of a healing response after inflammation and further stabilizes the atherosclerotic plaque. All these data indicate that molsidomine treatment is able to favour plaque stability in advanced atherosclerosis.

Interestingly, we also observed a lower spleen and kidney weight, indicative of decreased inflammation. Other research groups have demonstrated that molsidomine was able to reduce kidney weight [12], which was attributed to the anti-inflammatory and local dilating effects of NO. [44] In addition, molsidomine modulates inflammation in various conditions such as muscular atrophy [45,46], wound healing [47] and bacterial inflammation [48].

In conclusion, we demonstrated that administration of the NO donor molsidomine improves heart function, reduces neurological signs and enhances atherosclerotic plaque stability in a mouse model of vulnerable plaque and spontaneous myocardial infarction. The effect of molsidomine treatment on the phenotype of rupture-prone plaques in mice is new and might offer opportunities to further investigate the use of NO donors in atherosclerotic plaque stabilization and the prevention of cardiac and cerebral ischemia.

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Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2019.05.001.

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