Oral Magnesium Supplementation Induces Favorable Antiatherogenic Changes in ApoE-Deficient Mice

Hanne B. Ravn, Trine L. Korsholm, Erling Falk

Abstract—Epidemiological studies indicate that dietary magnesium influences atherogenesis. Magnesium inhibits plaque formation in animals receiving a high cholesterol diet, whereas the effect of magnesium in animals on low-fat diet has not been explored. Magnesium sulfate was given in the drinking water (50 mg/mL) to 7-week-old apolipoprotein E–deficient (apoE\(^{-/-}\)) mice (n=30). Control animals (n=30) received tap water. At the age of 19 weeks, the extent of atherosclerosis and the density of macrophages were measured in the aortic root, and blood lipids were analyzed. The median plaque area was significantly smaller in magnesium-treated female apoE\(^{-/-}\) mice and reached only 66% of control females (P<0.02). Plaque area was also less extensive in magnesium-treated male mice, although not statistically significant. Macrophage density was similar in both groups. Magnesium significantly reduced cholesterol (P<0.05) and triglyceride (P<0.01) levels, whereas high density lipoprotein cholesterol remained stable. No significant differences in body and heart weight were seen between treatment groups for either sex. In conclusion, in apoE\(^{-/-}\) mice receiving a low-fat diet, magnesium supplementation significantly inhibited atherogenesis in females but not males. Plaque composition remained unchanged in terms of macrophage density. This was obtained in association with significantly reduced levels of cholesterol and triglycerides. (Arterioscler Thromb Vasc Biol. 2001;21:858-862.)

Key Words atherosclerosis ■ mice ■ cholesterol ■ magnesium ■ macrophages

Epidemiological studies have indicated a relationship between magnesium content in drinking water and ischemic heart disease. The importance of magnesium has further been emphasized by autopsy studies demonstrating more coronary atherosclerosis and evidence of myocardial infarction in men from soft water areas as well as low magnesium concentrations in cardiac tissue in patients who died from sudden cardiac death. Also supporting the concept is the fact that experimentally induced magnesium deficiency changes the blood lipid composition in a more atherogenic direction. Moreover, it has already been shown that magnesium supplementation is able to reduce the cholesterol deposition in the vessel wall in rabbits as well as in mice fed a high cholesterol diet.

Murine models are increasingly used to study the possible mechanisms involved in atherogenesis. With the introduction of transgenic and gene-knockout techniques, a number of new murine strains have been developed with altered lipoprotein metabolism and atherosclerosis. Two of these murine models are known to generate extensive atherosclerosis throughout the entire arterial system, namely, the LDL receptor–deficient mice and the apoE-deficient (apoE\(^{-/-}\)) mouse. Severe hypercholesterolemia and mature atherosclerotic plaques can be induced in the LDL receptor–deficient mouse by a high-fat diet. In contrast, the apoE\(^{-/-}\) mouse spontaneously develops hypercholesterolemia and advanced atherosclerosis even on regular mouse chow.

Sherer et al have demonstrated retarded atherogenesis in LDL receptor–deficient male mice after magnesium fortification of the drinking water. In their study, the diet was not only high in cholesterol content, it was also supplemented with cholic acid. Some concern involving the nonphysiological nature and potential toxicity of cholate-containing diets has arisen, because the experimentally induced atherosclerosis may not accurately reflect the disease in humans.

The aim of the present study was to evaluate the effect of magnesium supplementation on atherogenesis in animals that did not receive a high-fat diet and/or cholic acid. For that purpose, apoE\(^{-/-}\) mice were randomized to tap water or water supplemented with magnesium sulfate. The diet consisted of regular mouse chow with normal magnesium content, to ensure that control animals would not develop magnesium deficiency.

Methods

Animals

Sixty homozygous apoE\(^{-/-}\) mice (C57BL/6 apoE\(^{-/-}\)) obtained from M&B A/S (Ry, Denmark) were kept in cages with a constant 12-hour day/12-hour night environment at 24°C and fixed humidity. After they were weaned, the mice were given regular chow (Altromin 1324, Altromin GmbH, Lage, Germany) and tap water until they reached only 66% of control females. In conclusion, magnesium supplementation significantly inhibited atherogenesis in females but not males. Plaque composition remained unchanged in terms of macrophage density. This was obtained in association with significantly reduced levels of cholesterol and triglycerides.
reached 7 weeks of age. The animals were then divided into 4 groups: 15 female mice (control females) and 15 male mice (control males) continued with tap water, and 15 female mice (magnesium-treated females) and 15 male mice (magnesium-treated males) were given water supplemented with magnesium sulfate at a final concentration of 50 mg/mL (≈6000 to 8000 mg/kg per day). The diet remained regular mouse chow throughout the study period, and diet and water were provided ad libitum. The animals were weighed 4 times during the study, at baseline and then once a month until the study was completed after 3 months. The animals were treated according to the principles stated in the Danish law on animal experiments.

**Lipid Analyses and P-Magnesium**

At the end of the study, the mice were anesthetized with an intraperitoneal injection of pentobarbital (150 mg/kg), the thorax was quickly opened, and the animals were exsanguinated by aspirating blood from their right ventricles. Nonfasting blood samples were obtained in precooled heparin-coated microtubes (Capiject T-MLH, Terumo Medical Corp) and immediately centrifuged at 3000 rpm at 4°C, and 200 μL plasma was stored at −20°C until analysis was performed. Total cholesterol and triglycerides were measured enzymatically on a Kone 30i analyzer with reagents from Labsystems OY. HDL cholesterol was measured in pooled plasma samples (n=2 in each group) by the precipitation method with Vitros 950 (Ortho-Clinical Diagnostics). Plasma magnesium (P-magnesium) levels were measured with the use of Vitros 950.

**Tissue Preparation and Quantification of Atherosclerosis**

The animals were perfused at ≈100 mm Hg with 4% phosphate-buffered formaldehyde (pH 7.2) via the left ventricle, which was then immersed in the fixative for 6 hours before cold storage (4°C) in PBS. The heart, including the ascending aorta, was removed, weighed, and paraffin-embedded. Sections of the aortic root were prepared by a slightly modified method of Paigen et al.12 The aortic root was sectioned serially at 4-μm intervals. The unstained sections were checked frequently by microscopic evaluation to identify the beginning of the aortic root. Once the aortic sinuses appeared, every other section was collected on glass slides. Five sections taken at 80-μm intervals, spanning 320 μm of the aortic root from the commissures of the aortic leaflets and upward, were stained with orcein and evaluated microscopically. Plaque area was measured blindly by the same person (T.L.K.) with the use of computer-assisted image analysis (Olympus BX50 light microscope, Sony DXC-151P color video camera, Imagraph Precision frame grabber, and SigmaScan Pro from Jandel Scientific Software). The amount of atherosclerosis in the aortic bulb was expressed as mean plaque size of the 5 sections.

**Macrophage Staining**

The amount of macrophages in plaques was assessed by immuno-staining for muramidase as previously described.13 Briefly, from each mouse a histological section from the level of maximum plaque size was deparaffinized, rehydrated, and incubated sequentially with pronase for 30 minutes (proteolytic antigen retrieval), blocking serum for 30 minutes (Vector S-1000, normal goat serum diluted 1:10), primary antibody overnight at 4°C (Dako A099, rabbit anti-muramidase, diluted 1:1000), secondary antibody for 30 minutes (Dako E0432, biotinylated goat anti-rabbit IgG, diluted 1:300), ABC-Complex/AP for 30 minutes (Dako K0376), substrate solution for 10 minutes (Vector SK-5100), and finally Mayer’s hematoxylin for 5 minutes (counterstaining). Controls were processed similarly, but the primary antibody was replaced with normal (nonimmune) rabbit IgG (Dako X0903, diluted 1:300) to ensure specificity. Macrophage-rich areas were measured by using SigmaScan Pro 4.0 from Jandel Scientific Software. When stained for muramidase, macrophages appear dark red. The relative area occupied by macrophages in each plaque was defined as the area (in pixels) within the plaque with a red scale level above a certain threshold (defined by the measurer). The threshold level was adjusted from section to section to accommodate the variation in staining intensity, and all measurements were carried out by 1 person (T.L.K.) unaware of sex and treatment (coefficient of variation 17.6%).

**Statistical Analysis**

Data are presented as median (interquartile range). Comparison was performed between groups by nonparametric analysis (Mann-Whitney test), and analysis was carried out for each sex individually. The relationship between variables was explored by the Spearman rank correlation. A value of P<0.05 was considered significant.

**Results**

**Body and Heart Weights**

Male mice were heavier than female mice, but there were no differences in body weights between magnesium-treated and control animals for either sex (Figure 1). Unsurprisingly, heart weight was higher in male mice than in female mice, but no difference was seen between the magnesium-treated and control groups for either sex (Table 1).

**Plaque Area in the Aortic Root**

The largest plaque areas were seen in female apoE−/− mice compared with male mice. The median plaque area in magnesium-treated females was only 66% (P<0.02) of that in control females (127 [range 98 to 163] versus 84 [range 67 to 107] ×10⁻³ mm², Figure 2). In males, the median plaque area was slightly lower, although not statistically significant in the magnesium-treated mice compared with the control mice (27 [range 20 to 40] versus 32 [range 22 to 53] ×10⁻³ mm², P=0.54; Figure 2). The plaque area did not show

| TABLE 1. Heart Weight in Magnesium-Treated and Control Mice |
|-----------------|-----------------|
|                  | Magnesium       | Control         |
| Male             | 0.21 (0.20–0.21)| 0.19 (0.18–0.23)|
| Female           | 0.16 (0.15–0.18)| 0.15 (0.15–0.16)|

Values are median (interquartile range).
any correlation with levels of total cholesterol or P-magnesium in any group.

**P-Magnesium and Lipid Analyses**

After magnesium supplementation, the P-magnesium levels increased significantly (by >50%) in male and female mice. Magnesium fortification in both sexes was associated with a significant reduction in plasma total cholesterol by 14%, and triglyceride levels were significantly reduced by 35% and 47% in females and males, respectively (Table 2). HDL cholesterol remained stable, as judged from the pooled plasma samples in each group (Table 2). P-magnesium showed no correlation with any of the lipid variables either in the control or in the treatment group.

**Plaque Composition**

Even though plaques were significantly smaller in magnesium-treated females, the macrophages occupied an equal area relatively within the plaque (Figure 3). The macrophage-rich area in percentage of total plaque area did not differ between treatment groups either in female or male mice (Table 3). The fractional macrophage-rich area was significantly higher in female mice (27% [range 20% to 34%]) compared with male mice (18% [range 11% to 28%], P<0.05).

**Discussion**

The present study shows that oral magnesium supplementation induces beneficial antiatherogenic effects in apoE^{-/-} mice fed low-fat regular mouse chow. The atherosclerotic plaque area in the aortic root was less extensive in mice receiving magnesium supplementation, although the difference was only statistically significant in female mice. Significant reductions in cholesterol and triglyceride levels were observed in female and male mice.

**Figure 2.** Atherosclerosis in aortic root. Plaque area is shown in control female apoE^{-/-} mice (solid circle), magnesium-treated female apoE^{-/-} mice (open circle), control male apoE^{-/-} mice (solid inverted triangle), and magnesium-treated male apoE^{-/-} mice (open inverted triangle). Median plaque area is displayed by the bar in each treatment group.

**Figure 3.** Immunohistochemical staining with muramidase was used for identification of macrophages (dark red area). Plaque area was significantly smaller in magnesium-treated female apoE^{-/-} mice (top) compared with control mice (bottom), but macrophages occupied a similar percentage of total plaque area.

**TABLE 2. Cholesterol, Triglyceride, and P-Magnesium Levels in Magnesium-Treated and Control Mice**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Magnesium</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12.0 (11.6–13.6)</td>
<td>10.5 (9.6–11.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>11.2 (10.6–12.2)</td>
<td>9.9 (8.7–11.5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.65 (1.34–1.83)</td>
<td>1.12 (0.95–1.33)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Female</td>
<td>0.88 (0.8–1.06)</td>
<td>0.65 (0.6–0.71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.50</td>
<td>0.60</td>
<td>...</td>
</tr>
<tr>
<td>Female</td>
<td>0.25</td>
<td>0.40</td>
<td>...</td>
</tr>
<tr>
<td>P-magnesium, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.07 (1.02–1.16)</td>
<td>1.78 (1.67–1.92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>1.15 (1.09–1.19)</td>
<td>1.76 (1.44–1.83)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are median (interquartile range). HDL cholesterol was measured on pooled plasma samples (n=2 in each group).

**TABLE 3. Macrophage Density in Magnesium-Treated and Control Mice**

<table>
<thead>
<tr>
<th></th>
<th>Macrophage Density, % of Total Plaque Area</th>
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<tbody>
<tr>
<td>Magnesium</td>
<td>Control</td>
</tr>
<tr>
<td>Male</td>
<td>17 (11–24)</td>
</tr>
<tr>
<td>Female</td>
<td>33 (21–35)</td>
</tr>
</tbody>
</table>

Values are median (interquartile range).
The relationship between water hardness and mortality of ischemic heart disease has been explored during the past 3 decades. Several epidemiological studies have shown an inverse relationship between cardiovascular mortality and the magnesium content in drinking water.\textsuperscript{1,3} That a causal relationship between magnesium and ischemic heart disease should exist has been argued as being biologically plausible. Supporting this concept, epidemiological surveys have documented a dose-response relationship, with declining occurrences of ischemic heart disease as the magnesium level in drinking water increases.\textsuperscript{15} It is not known whether the association is due to a deficiency state in those who consume low dietary magnesium or whether magnesium exerts its benefit in association with an adequate diet. In the present study, the control animals received standard mouse chow with a sufficient magnesium content, and the observed effect can therefore be ascribed to what can be achieved by giving magnesium as a dietary supplement.

The effect of dietary magnesium on the atherogenic process has been evaluated in several animal experiments. Rabbits fed a high cholesterol diet supplemented with a high magnesium load were shown to have lower levels of cholesterol and triglycerides and less extensive atherosclerotic lesions.\textsuperscript{4} In the study by Renaud et al.,\textsuperscript{16} magnesium substitution lowered cholesterol levels, but the severity of atherosclerosis in the aorta was insignificantly inhibited. In contrast, Orimo and Ouchi\textsuperscript{17} found significantly retarded atherosclerosis but unaltered lipid levels. The reason for the discrepancy between the first and the 2 latter rabbit studies may be related to the fact that much lower amounts of magnesium were given in the latter 2 studies, resulting in a more inconsistent effect. Furthermore, magnesium was administered in the drinking water in the first study but as part of the diet in the 2 latter studies. It has been argued that the bioavailability of magnesium is higher from water than from food,\textsuperscript{15} which in theory would also contribute to a reduced effect.

The effect of magnesium supplementation has previously been evaluated in mice. Four different doses of magnesium were added to the drinking water in nontransgenic (ICR) male mice on a synthetic atherogenic diet. The lowest magnesium intake, equivalent to magnesium deficiency, resulted in the highest serum cholesterol level, and the remaining 3 groups had cholesterol levels inversely related to the magnesium intake.\textsuperscript{5} No effect was observed for triglyceride or HDL cholesterol levels, but magnesium intake prevented cholesterol deposition in the aorta.\textsuperscript{5} In LDL receptor–deficient mice, magnesium fortification of the drinking water did not affect the lipid profile but resulted in less atherosclerosis in the aortic root in male but not female mice.\textsuperscript{6} In contrast, significant inhibition of atherosclerosis was seen only in the female apoE\textsuperscript{2/2} mice in the present study. The variability in inhibition of atherogenesis is not likely to reflect sex differences in the response to magnesium therapy but probably reflects the variation in plaque development. Female apoE\textsuperscript{2/2} mice exhibit mature fibrofatty atherosclerotic plaques as early as 16 weeks of age, and they have significantly larger lesions compared with age-matched males.\textsuperscript{18} However, there is a catch-up by males, and at the age of 48 weeks, lesion size is equivalent to that in the females or even larger.\textsuperscript{6,18} In contrast, atherosclerotic lesions in the LDL receptor–deficient mouse are larger in males than in females, which has been shown repetitively in several studies.\textsuperscript{5,8,19,20} Retarded atherogenesis was observed in female LDL receptor–deficient mice and in male apoE\textsuperscript{2/2} mice after magnesium supplementation, but data did not reach the level of significance, which is probably due to the inherent slow progression of atherosclerosis.

It has become evident during the last decade that inflammation plays an important role in atherogenesis. Monocytes, macrophages, and cytokines play a key role in the initiation and progression of atherosclerotic plaques.\textsuperscript{21} Magnesium deficiency in rats has been associated with increased levels of macrophage-derived cytokines.\textsuperscript{22,23} Altura et al\textsuperscript{24} have shown that magnesium-deficient rabbits on high cholesterol diets have increased macrophage activity and that magnesium supplementation significantly reduces the activity. In contrast, the percentage of macrophage-rich areas to total plaque area was not significantly different between treatment groups in the present study. Supporting these findings, a study by Shechter et al\textsuperscript{24} has demonstrated that oral magnesium supplementation in patients with coronary heart disease does not induce significant changes in the expression of surface adhesion molecules on monocytes, except for a reduction in the MAC-1 antigen. To visualize macrophages in plaques, we used a polyclonal antibody raised against human muramidase, which cross-reacts with rodent muramidase (for a list of references, see Falk et al\textsuperscript{12}). Endothelial cells, lymphocytes, smooth muscle cells, and platelets are muramidase negative. Normal blood monocytes, resident tissue macrophages, and activated macrophages may contain muramidase, particularly the latter, and muramidase is constitutively produced by monocytes/macrophages in culture.\textsuperscript{12} There were significant differences in the percentage of macrophage-rich areas between sexes, indicating intense inflammation and growth in female plaques at this stage.

The mechanism(s) behind the antiatherogenic effect of magnesium remains to be elucidated. It has been argued that magnesium and other divalent cations have the ability to form insoluble salt complexes with fatty acids or to form complexes with bile acid derivatives and in this way reduce the lipid uptake.\textsuperscript{4,16} In the present study, the animals were on low-fat chow with a diminished exogenous contribution of cholesterol, eliminating reduced absorption as a possible explanation. Magnesium has been used as a laxative for years, and increased excretion of nutrients could also interfere with the development of atherosclerosis, but the similar growth curves for both sexes indicate that all animals thrived independently of treatment.

In apoE\textsuperscript{2/2} mice, a high-fat Western-type diet elevates the plasma cholesterol level markedly (up to 5-fold higher levels versus chow diet) and accelerates the development of atherosclerosis, which is why it is generally assumed, but not proven, that lesion size in this murine model of atherosclerosis is dependent on the cholesterol level.\textsuperscript{5,25} However, in chow-fed control mice, only 1 study has shown a statistically significant correlation between plasma cholesterol and en face atherosclerosis in the aorta but not between plasma cholesterol and lesion size in the aortic root.\textsuperscript{26} Thus, there is only a weak, if any, relationship between plasma cholesterol and atherosclerosis in chow-fed apoE\textsuperscript{2/2} mice, and the very modest difference in lipid values in the present study is not an acceptable explanation for the inhibition of atherogenesis in magnesium-supplemented mice.
Increased extracellular magnesium concentrations have been shown to blunt the release of free radicals after ischemia/reperfusion injury, indicating antioxidative properties. Apart from playing a key role in arterial progression, reduction in platelet reactivity would thus reduce plaque formation. Apart from playing a key role in arterial progression. Apart from playing a key role in arterial progression. Apart from playing a key role in arterial progression.

In accordance, lipid peroxide levels were significantly decreased, and cholesterol deposition in the aorta was reduced in mice receiving magnesium supplementation. Finally, magnesium is known to reduce platelet reactivity in vitro and ex vivo after intravenous infusion. Recently, it has been shown that oral magnesium supplementation is also able to reduce platelet-dependent thrombosis in patients with coronary artery disease. Apart from playing a key role in arterial progression, platelets are also involved in atherogenesis, a condition in which they (in combination with the activated endothelium and macrophages) release growth factors, which stimulate smooth muscle cells to proliferate, migrate, and synthesize new matrix. In mature plaques, endothelial denudation with platelet adhesion may contribute to lesion growth (platelet monolayer–releasing growth factors). A reduction in platelet reactivity would thus reduce plaque progression.

In conclusion, the present study shows a beneficial antiatherogenic effect of magnesium supplementation in the drinking water. In female apoE mice, a significant reduction in the atherosclerotic burden in the aortic root was observed; the plaque area was also smaller in magnesium-treated male mice, although data did not reach statistical significance. Whether the benefits of magnesium supplementation can be transferred to humans is at present unknown. Further studies are warranted to elucidate the role of magnesium in the development of atherosclerosis and ischemic heart disease.

Acknowledgments
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References
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