Increased Atherosclerotic Lesion Calcification in a Novel Mouse Model Combining Insulin Resistance, Hyperglycemia, and Hypercholesterolemia

Suvi E. Heinonen, Pia Leppänen, Ivana Kholová, Henri Lumivuori, Sanna-Kaisa Häkkinen, Fatima Bosch, Markku Laakso, Seppo Ylä-Herttuala

Abstract—No mouse model is currently available where the induction of type 2 diabetes on an atherosclerotic background could be achieved without significant concomitant changes in plasma lipid levels. We crossbred 2 genetically modified mouse strains to achieve a model expressing both atherosclerosis and characteristics of type 2 diabetes. For atherosclerotic background we used low-density lipoprotein receptor–deficient mice synthetizing only apolipoprotein B100 (LDLR−/− ApoB100/100). Diabetic background was obtained from transgenic mice overexpressing insulin-like growth factor-II (IGF-II) in pancreatic beta cells. Thorough phenotypic characterization was performed in 6- and 15-month-old mice on both normal and high-fat Western diet. Results indicated that IGF-II transgenic LDLR−/− ApoB100/100 mice demonstrated insulin resistance, hyperglycemia, and mild hyperinsulinemia compared with hypercholesterolemic LDLR−/− ApoB100/100 controls. In addition, old IGF-II/LDLR−/− ApoB100/100 mice displayed significantly increased lesion calcification, which was more related to insulin resistance than glucose levels, and significantly higher baseline expression in aorta of several genes related to calcification and inflammation. Lipid levels of IGF-II/LDLR−/− ApoB100/100 mice did not differ from LDLR−/− ApoB100/100 controls at any time. In conclusion, type 2 diabetic factors induce increased calcification and lesion progression without any lipid changes in a new mouse model of diabetic macroangiopathy. (Circ Res. 2007;101:1058-1067.)

Key Words: type 2 diabetes ■ atherosclerosis ■ insulin resistance ■ mouse model ■ hypercholesterolemia

Type 2 diabetes is a heterogenous disorder characterized by peripheral insulin resistance (IR) and β-cell failure. Individuals with IR and a resulting impaired glucose tolerance as well as with overt type 2 diabetes have an increased predisposition to atherosclerosis and related cardiovascular diseases,1 which are also the major causes of mortality in these patients.2

It has remained unclear whether induction of type 2 diabetes in atherosclerosis-prone mice can be attained in the absence of altered lipid levels.3 In humans, diabetes itself does not generally lead to marked elevations in total cholesterol. However, in most animal models, induction of diabetes causes major concomitant changes in lipid values.3 Thus, it has been very difficult to differentiate the effects of diabetic factors from those of hyperlipidemia, especially in mice where hypercholesterolemia is clearly the most essential factor for the development of atherosclerotic lesions.3

The low-density lipoprotein receptor–deficient mice able to synthesize only apolipoprotein B100 (LDLR−/− ApoB100/100) represent a model of hypercholesterolemia with elevated levels of LDL cholesterol and expression of only apolipoprotein B100. This leads to accelerated atherogenesis4 and a lipid profile, which resembles the type commonly found in human hypercholesterolemia and atherosclerosis5 better than any other mouse model currently available. Therefore, the LDLR−/− ApoB100/100 mice are well suited to experimental atherosclerosis studies because they better resemble human situation than, eg, the apolipoprotein E–knockout mice (ApoE−/−),6,7 which have a very different remnant-like lipoprotein pattern compared with humans and lack all functions of apolipoprotein E on cholesterol homeostasis, cellular cholesterol efflux and inflammatory responses.8

We cross-bred the atherogenic LDLR−/− ApoB100/100 mice with transgenic mice in which type 2 diabetes is caused by overexpressing insulin-like growth factor-II (IGF-II) in pancreatic beta (β) cells.9 A model was generated which manifests characteristics of type 2 diabetes with no major changes in the plasma lipoprotein fractions while still showing worsening of the macrovascular lesion phenotype.

Materials and Methods
Mice deficient of LDL receptor and expressing only apolipoprotein B100 (LDLR−/− ApoB100/100) in C57BL/6x129/SvJae background...
The Jackson Laboratory, Bar Harbor, Me) were crossbred with C57BL6/SJL mice overexpressing IGF-II in pancreatic H9252 cells for 10 generations. IGF-II negative LDLR/H11002/ApoB100/100 littermates served as controls. Both 6- and 15-month-old mice were examined either so that they were fed with a normal chow diet (R36, Lactamin) for the whole time or a high fat, Western diet (TD 88173, Harlan Teklad: 42% of calories from fat and 0.15% from cholesterol, no sodium cholate) for the last 3 months. Both female and male mice were used in this study, and the study groups contained equal numbers of both sexes. Experiments were approved by the Experimental Animal Committee of the University of Kuopio.

For further details and other methods, see Materials and Methods data supplement available at http://circres.ahajournals.org.

Results
Insulin Resistance and Hyperglycemia in the IGF-II/LDLR−/−ApoB100/100 Mice

Intraperitoneal glucose (GTT) and insulin tolerance tests (ITT) were performed to investigate glucose intolerance and insulin resistance. In the GTT a sustained elevation of blood glucose level was observed in IGF-II/LDLR−/−ApoB100/100 mice. This finding was evident on both normal (Figure 1B) and Western diet (Figure 1A), which suggests that the IGF-II/LDLR−/−ApoB100/100 mice are glucose-intolerant independently of the diet. To examine insulin secretion in response to intraperitoneal glucose injection during the GTT, plasma insulin levels were determined at different time points in the group on Western diet (Figure 1C). In contrast to LDLR−/−ApoB100/100 controls, despite being hyperinsulinemic in the beginning, an absent acute first phase insulin response after glucose stimulation was observed in the IGF-II/LDLR−/−ApoB100/100 mice and their insulin levels remained almost unchanged from 60 to 120 minutes. Furthermore, in the ITT a significantly reduced insulin sensitivity after insulin administration was observed in the IGF-II/LDLR−/−ApoB100/100 group on normal diet (Figure 1D).

Diabetic Factors Do Not Cause Changes in Lipid Levels but Raise the Basal Osteoprotegerin Level

Metabolic parameters are presented in Table 1. Glucose levels of 6-month-old IGF-II/LDLR−/−ApoB100/100 mice were significantly elevated on normal diet both in fed and fasted states. The effect of Western diet on fasting glucose levels was larger in the group of IGF-II/LDLR−/−ApoB100/100 mice: glucose levels were notably higher compared with LDLR−/−ApoB100/100 controls (7.5 ± 2.1 versus 4.7 ± 1.5 mmol/L, P < 0.05) and 16% of the IGF-II/LDLR−/−ApoB100/100 mice demonstrated fasting glucose values over 10 mmol/L versus none of the LDLR−/−ApoB100/100 mice. Similar findings were also seen in 15-month-old animals on Western diet, although differences on normal diet had disappeared. On normal diet, plasma insulin levels of fed IGF-II/LDLR−/−ApoB100/100 mice were twice as high as those of the LDLR−/−ApoB100/100 controls (2.1 ± 0.5 versus 1.0 ± 0.1 ng/mL, respectively,
differences in body weights were detected neither on normal Western diet the OPG level was equally high in the OPG levels than did the control females (P<0.05). Except for the trend that IGF-II/LDLR−/− A poB100/100 females seemed to gain more weight than did the control females, no significant differences in body weights were detected neither on normal nor Western diet and therefore the elevation of insulin levels in IGF-II/LDLR−/− A poB100/100 mice cannot be explained by weight change. Combination of mild hyperglycemia with elevated insulin levels in the IGF-II/LDLR−/− A poB100/100 mice indicates insulin resistance, leading to even more severe phenotype after 3 months of Western diet.

Severe hypercholesterolemia developed on Western diet both in IGF-II/LDLR−/− A poB100/100 and LDLR−/− A poB100/100 mice without any differences between the sexes (Table 1). Similar trend was observed in fasting free fatty acids levels (FFA), whereas no changes were present in fasting triglycerides. Thus, diabetic factors present in the IGF-II/LDLR−/− A poB100/100 mouse did not cause any detectable changes in the lipid metabolism. Interestingly, 15-month-old IGF-II/LDLR−/− A poB100/100 mice had a significantly higher plasma osteoprotegerin (OPG) level than LDLR−/− A poB100/100 controls on normal diet. In hypercholesterolemic state on Western diet the OPG level was equally augmented in both groups. However, despite the general elevation, IGF-II/LDLR−/− A poB100/100 females still displayed higher OPG levels than did the control females (P=0.019, data not shown).

Overall Lesion Development Is Not Affected by Diabetic Factors

Atherosclerosis was quantified both en face from the whole aorta and from serial cross-sections from the aortic sinus level. In the en face analysis the distribution of macroscopic lesions in 6-month-old mice was similar in both groups and they occurred at typical sites for LDLR−/− mice exposed to prolonged hypercholesterolemia. There were no differences in the overall area of macroscopic lesions between 6-month-old IGF-II/LDLR−/− A poB100/100 mice and LDLR−/− A poB100/100 controls neither on normal (14.0±3.6% versus 14.3±5.2%, respectively) nor on Western diet (14.4±3.8% versus 16.6±3.5%, respectively; Figure 2A). Hence, the mild hyperglycemia observed in the IGF-II/LDLR−/− A poB100/100 mice did not have a direct effect on the lesion area, and no significant correlation was seen between fasting glucose levels and lesion areas (Figure 2B). However, on Western diet there was a strong correlation between the extent of lesions and fed state insulin levels in IGF-II/LDLR−/− A poB100/100 mice (r=0.74, P<0.05), which was not detectable in LDLR−/− A poB100/100 controls (r=0.56, NS; Figure 2C).

Although the overall en face lesion area of 6-month-old mice was not considerably affected by Western diet, thickness of the lesions increased significantly in both groups after three months of Western diet as seen in the cross-sectional analysis. Compared with mice on normal diet, lesion size increased by 5.1-fold in IGF-II/LDLR−/− A poB100/100 mouse from 9.6±6.0% to 49.2±5.2%, P<0.0001 and by 2.9-fold in LDLR−/− A poB100/100 controls (from 17.3±9.2% to 49.5±8.9%, P<0.0001; Figure 2D). Aging also enhanced lesion development: compared with 6-month-old animals the average cross-sectional lesion area was more than doubled in 15-month-old mice on normal diet (35.6±6.9% in IGF-II/LDLR−/− A poB100/100 mice and 39.5±8.6% in controls, P<0.01 in both groups; Figure 2E). Nevertheless, Western diet further increased the lesion size also in older animals with cross-sectional areas of 52.2±9.2% in IGF-II/LDLR−/− A poB100/100 mice (P<0.01) and 56.8±8.0% in the LDLR−/−
ApoB^{100/100} group (P<0.001; Figure 2E). However, no differences in the final lesion areas were found between the groups on normal or Western diet.

**Increased Calcification and Accelerated Lesion Progression in IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) Mice**

To investigate the role of aging and diet on the development of lesions, composition of the lesions was examined from aortic cross-sections from both 6- and 15-month-old mice. In younger animals on normal diet the lesions mainly consisted of macrophage-derived foam cells (Figure 3A and 3B). On Western diet (Figure 3C and 3D) the amount of cholesterol crystals was increased over 2-fold in both groups and calcification was found on normal diet, even though the lesion sizes and fasting glucose levels in 6-month-old mice (Figure 3E).

Cross-sectional lesion areas from the aortic sinus level in 6-month-old mice (D) and in 15-month-old mice (E) on normal and Western diet. B, There was no correlation between en face lesion areas and fasting glucose levels in 6-month-old mice on Western diet (r=0.51, P=NS in LDLR\(^{-/-}\)ApoB\(^{100/100}\) controls and r=0.54, P=NS in IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice). C, However, a significant correlation was observed with the en face lesion areas and fed state plasma insulin in IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice (r=0.74, P<0.05) but not in the LDLR\(^{-/-}\)ApoB\(^{100/100}\) controls (r=0.56, P=NS). Cross-sectional lesion areas from the aortic sinus level in 6-month-old mice (D) and in 15-month-old mice (E) on normal and Western diet.

Plaque calcification did not correlate significantly with fasting glucose levels (Figure 4E). However, plotting calcification against the incremental glucose areas under the curve (AUC) in a GTT suggested a connection between these parameters (Figure 4F). Hence, it seems that in this model the factors stimulating intimal calcification are more evidently related to insulin resistance than to hyperglycemia.

**Mechanisms of Increased Calcification**

To study the mechanisms behind increased calcification, we first examined the expression of different molecules in the lesions by immunohistochemical methods in 6- and 15-month-old mice on both diets (Table 2). We observed intensity changes in the stainings attributed to aging or diet without any significant differences between the IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice and LDLR\(^{-/-}\)ApoB\(^{100/100}\) controls. In the assessment of angiogenic markers in the lesions only a weak VEGF signal was detected in both groups, mostly localizing in macrophages and also sparsely in the medial smooth muscle cells (Figure 5A and 5B). These cell types, together with the endothelium, demonstrated an intense calcified areas and cholesterol clefts were combined to assess the total size of the necrotic core, IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice demonstrated significantly higher percentages than LDLR\(^{-/-}\)ApoB\(^{100/100}\) controls (38.0±16.0% versus 16.6±13.0%, P<0.05; Figure 4D). In addition, the IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice also manifested a more advanced lesion phenotype with less organized structure and focal thinning of the fibromuscular cap (Figure 3G and 3H).
RAGE immunoreactivity as well (Figure 5C and 5D). Groups also showed equal lesional macrophage contents (data not shown) and similar expression patterns with inflammatory markers such as VCAM-1 (Figure 5E and 5F), ICAM-1 (Figure 5G and 5H), and NFkB (Figure 5I and 5J). No quantifiable differences in the expression of eNOS (Figure 5K and 5L), OPN (Figure 5M and 5N), oxidized LDL, neovascularization, proliferation or apoptosis (data not shown) in the lesions could be detected by immunohistochemistry.

We next examined the expression levels of selected genes in tissue samples of intact aorta and lesions from 15-month-old mice on Western diet with quantitative real-time RT-PCR. Results revealed that the baseline expression of genes related to calcification (OPN, ALP-2 and BMP-2, Figure 6A through 6C) and inflammation (MCP-1, Figure 6E) as well as scavenger receptor CD36 (Figure 6D) were higher in IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice than in normal aortas of hypercholesterolemic LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> controls. No statistical differences were found between the groups in the expression of Bax (Figure 6G) or IL-6 (Figure 6F), although there was a trend toward a higher IL-6 expression in lesions of the IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice and the change seen in lesional Bax expression was similar to that found in human studies of carotid lesions.11

**Figure 3.** Changes in lesion composition in relation to age and diet in IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice (left panel) and LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> controls (right panel). Modified Movat’s pentachrome staining from aortic arch sections of 6-month-old mice (A–D) and 15-month-old mice (E–H) either on normal diet (A, B, E, and F) or after 3 months of Western diet (C, D, G, and H). Original magnifications ×200 (A–F), ×100 (G and H). Scale bars 50 μm.

**Discussion**

Studying diabetic angiopathy and cardiovascular complications related to type 2 diabetes in vivo has been difficult because of the lack of good animal models. In the present study we examined the effects of insulin resistance and hyperglycemia on the atherogenic background LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> demonstrating an atherogenic lipoprotein profile resembling that of humans. By cross-breeding these mice with type 2 diabetic IGF-II transgenic mice, we achieved a
Figure 4. Evaluation of plaque calcification in 15-month-old mice on Western diet. A, Aortic cross-sections of IGF-II/LDLR\(^{+/−}\) ApoB\(^{100/100}\) and B, LDLR\(^{+/−}\) ApoB\(^{100/100}\) control mice stained with alizarin red S (magnification \(×100\), scale bar 50 \(\mu\)m). C, Quantification of calcified area from the total plaque area. D, Size of the necrotic core of the plaque with calcified area and cholesterol clefts combined. Plaque calcification in relation to fasting glucose (E) and insulin resistance (expressed as glucose AUC values acquired from glucose tolerance test; F).

Table 2. Expression of Different Molecules in Atherosclerotic Lesions of 6- and 15-Month-Old IGF-II/LDLR\(^{+/−}\) ApoB\(^{100/100}\) Mice and LDLR\(^{+/−}\) ApoB\(^{100/100}\) Controls on Normal and Western Diet Determined by Immunohistochemical Methods

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− Indicates no detectable staining; +/−, some positivity; +, weak staining; ++, moderate staining; ++++, strong staining; ND, not determined.
promising atherosclerosis model expressing type 2 diabetic features with no additional changes in hyperlipidemia but, nevertheless, worsening of the lesion phenotype.

Clinical trials show that the treatment of hyperglycemia in type 2 diabetes does not reduce macrovascular complications. Therefore choosing an appropriate animal model for studies on the effects of diabetes on atherosclerosis is crucial. So far, attempts to generate mouse models for type 2 diabetes and atherosclerosis have usually been based either on feeding the atherosclerosis-prone LDLR\(^{-/-}\) or ApoE\(^{-/-}\) mice different diets or crossing them with models of obesity and type 2 diabetes, such as leptin-deficient (ob/ob) or leptin receptor-deficient (db/db) mice. However, the main difficulty with dietary induction of type 2 diabetes has been that the effects vary considerably depending on the mouse model and especially on the duration and composition of the diet. Generally, the changes in glucose metabolism have also been quite modest, thus resulting in a situation where severe hypercholesterolemia usually masks their possible contribution to the lesion development. This has been the case also for crossings with models of disturbed leptin metabolism, because parallel to glucose and insulin levels also total cholesterol is significantly increased in LDLR\(^{-/-}\)ob/ob, ApoE\(^{-/-}\)ob/ob and ApoE\(^{-/-}\)db/db mice compared with the respective LDLR\(^{-/-}\) or ApoE\(^{-/-}\) controls. Therefore, the main driving force of lesion progression in these models is probably still increased cholesterol level, supported by the observations that no lesions developed when ob/ob mice were crossed with LDLR\(^{-/-}\) mice having a markedly lower cholesterol level and that the plain diabetic IGF-II transgenic mice do not develop atherosclerotic lesions if cholesterol levels are low (Heinonen et al, unpublished observation). In our study the background models were chosen to avoid disturbances in the levels and functions of apolipoprotein E and leptin, because they both have important and complex roles in physiology. In addition, instead of using only relatively young animals, we examined also old mice to investigate the effects of diabetes on advanced lesions and to mimic physiological conditions of an average type 2 diabetes patient with cardiovascular complications.

In the present study both IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice and LDLR\(^{-/-}\)ApoB\(^{100/100}\) controls developed atherosclerotic lesions covering about 15% of the total aortic area. This finding is in line with other studies performed with the LDLR\(^{-/-}\)ApoB\(^{100/100}\) model. Hypercholesterolemia induced by Western diet clearly thickened the plaques in both groups and led to the development of more advanced lesions with macrophage infiltrates and large necrotic areas covered by a fibrous cap. Nevertheless, the observed association between en face lesion area and fed state insulin level in the IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice supports the proatherogenic effect of the prediabetic state. In addition, compared with the
LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> controls, IGF-II/LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> mice on Western diet and especially in the group of old mice presented clearly less organized and more complex lesions with significantly increased calcification. Larger necrotic cores<sup>22,23</sup> and increased calcification<sup>23</sup> in coronary plaques have been reported also in type 2 diabetes patients with sudden coronary death. In fact, the presence of radiologically detectable calcification in peripheral arteries,<sup>24</sup> coronaries, or abdominal aorta<sup>25</sup> are all reported to be strong markers of future cardiovascular events in patients with type 2 diabetes. Our findings that the calcification was most profound in old animals is consistent with clinical studies, where age and duration of diabetes have remained as independent risk factors for coronary artery calcification in type 2 diabetics.<sup>26,27</sup>

There are several possible mechanisms which could cause increased calcification in type 2 diabetes. For example, AGE formation has been connected to vascular calcification.<sup>28</sup> However, in our study aortic RAGE expression was equal in both groups and thus the AGE-RAGE interaction probably does not explain increased calcification. This is not surprising, because hypercholesterolemia makes the vasculature of also nondiabetic mice susceptible to oxidant stress, inflammation and generation of AGEs. In support of this notion, soluble RAGE administration has been reported to attenuate the lesion area and complexity also in normoglycemic hypercholesterolemic mice.<sup>29</sup> RAGE activation has also been suggested to stimulate VEGF expression in atherosclerotic plaques.<sup>30,31</sup> Thus, uniform RAGE expression between the groups might account for the similarity of VEGF expression and the subsequent lack of differences in intraplaque angiogenesis as well. The presence of several other molecules in atherosclerotic lesions was also examined using immunohistochemical methods. Moderate expression of eNOS and NFκB was detected in lesions of old IGF-II/LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> and LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> mice, whereas intense stainings for VCAM-1, ICAM-1, and OPN were found in lesions of both diabetic and nondiabetic mice. However, none of these findings were able to explain increased calcification of lesions in the diabetic IGF-II/LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> mice, probably because the potential underlying differences are beyond the sensitivity of immunohistochemistry. When quantitative RT-PCR was used for the analysis of candidate gene expression in normal aortas and atherosclerotic lesions we found much higher baseline expression levels of all calcification-related genes (OPN, ALP-2, and BMP-2) in newborn aortas of IGF-II/LDLR<sup>−/−</sup> ApoB<sub>100/100</sub> mice. The role of these factors in diabetic vascular calcification has been widely recognized.<sup>32–34</sup> The same profile was observed in scavenger receptor CD36 expression which is in line with human studies<sup>35</sup> and suggests a proatherogenic mechanism through increased macrophage uptake of LDL.
expression levels of MCP-1 and IL-6 suggest a trend toward a more inflammatory environment in the lesions of IGF-II/LDLR−/−apoB100/100 mice. When also higher plasma OPG levels of the IGF-II/LDLR−/−apoB100/100 mice are taken into account, it seems likely that no currently known single factor is solely responsible for the increased calcification but accelerated lesion progression is rather a net result of several factors upregulated in the diabetic aorta.

In conclusion, the IGF-II/LDLR−/−apoB100/100 model generated in this study demonstrates significantly increased atherosclerotic calcification and complexity of atherosclerotic lesions in older animals, and thus represents a very promising new model for studies of macrovascular complications in type 2 diabetes.

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Disclosures
None.

References


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MATERIALS AND METHODS

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Materials and Methods

Animals

Mice deficient of LDL receptor and expressing only apolipoprotein B100 (LDLR\(^{-/-}\) ApoB\(^{100/100}\), genetic background \(\sim 75\%\) C57BL/6 and \(\sim 25\%\) 129/SvJae)\(^1\) were obtained from the Jackson Laboratory and crossbred with C57BL6/SJL mice overexpressing IGF-II in pancreatic \(\beta\)-cells\(^2\) for ten generations. IGF-II negative LDLR\(^{-/-}\)ApoB\(^{100/100}\) littermates served as controls. Mice were fed ad libitum with either a normal chow diet (R36, Lactamin, Sweden) for six months (\(n=30\)) or a high fat, Western diet (TD 88173, Harlan Teklad: 42 \% of calories from fat and 0.15 \% from cholesterol, no sodium cholate) for three months (\(n=30\)) so that the mice in both diet groups were 6-7 months old at the time of analyses. In addition we examined older animals (age 15 months in both diet groups) on normal diet (\(n=13\)) and after three months on Western diet (\(n=18\)). Both female and male mice were used in this study and the groups consisted of both sexes equally. During experiments mice were anesthetized using fentanyl-fluanisone (3.15 and 10 mg/kg) / midazolam (5 mg/kg) subcutaneously and euthanized using carbon dioxide. Mice were housed in groups and maintained in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle at the National Laboratory Animal Centre in Kuopio. Experiments were approved by the Experimental Animal Committee of the University of Kuopio.

Metabolic Analyses

Glucose tolerance tests (GTT) were carried out in mice fasted overnight (15 h). Animals received an intraperitoneal glucose injection (1.5 g/kg) and blood glucose levels were measured from tail tip right before the injection and at time-points 30, 60, 90 and 120 min. For insulin tolerance tests (ITT) mice were injected i.p. with a solution of insulin (0.5 IU/kg
[Ultratard, Novo Nordisk A/S]) and blood glucose was measured before and 5, 15, 30, 45 and 60 min after the injection. Blood glucose was determined using a Glucometer Elite analyzer (Bayer). Plasma insulin levels were measured using an ELISA kit (Rat/mouse Insulin ELISA Kit, Linco Research Inc.). Triglycerides, total cholesterol and free fatty acids were determined from overnight fasting plasma samples as described previously. Osteoprotegerin levels were measured from fasting plasma samples with an ELISA kit (Mouse OPG/TNFSR5F11B Immunoassay, Quantikine, R&D Systems).

**Histological Evaluation of Atherosclerosis**

Mice were sacrificed and perfused with phosphate-buffered saline and 4% paraformaldehyde (PFA) (pH 7.4). Aortas were either embedded in paraffin for cross-sectional analysis or the adventitia was removed and the aortas were opened longitudinally and pinned out for *en face* evaluation. Percentage of *en face* lesions in the whole aorta and cross-sectional lesion areas from the sinus level were quantified as described. Cholesterol clefts and calcification were measured from hematoxylin-eosin stained sections as a percentage from the respective plaque area. Analyses were performed in a blinded fashion using AnalySIS software (Soft Imaging System GmbH). Immunohistochemical stainings were performed on serial 6 µm sections as described. For the analysis of atherosclerotic lesions, serial cross-sections cut from the aortic arch were stained with a modified Movat’s pentachrome stain, Alizarin Red S or immunostained with the following antibodies: macrophages (mMQ AIA31240, 1:5000, Accurate Chemical & Scientific Corp.), oxidation-specific epitopes (MAL-2, 1:1000), smooth muscle cells (α-actin, 1:200, clone 1A4, Sigma-Aldrich Corp.), CD31 (platelet endothelial cell adhesion molecule [PECAM]-1, 1:50; BD Biosciences Pharmingen), proliferating cell nuclear antigen (PCNA, 1:500, clone PC10, NeoMarkers, Lab Vision Ltd.), caspase-3 (1:250, Promega Corp.), osteopontin (OPN, 1:500, R&D Systems), receptor for
advanced glycation end products (RAGE, 1:50, R&D Systems), mouse vascular endothelial growth factor A (mVEGF-A, 1:500, Serotec), endothelial nitric oxide synthase (eNOS, 1:150, BD Biosciences Pharmingen), nuclear factor kappa B (NfκB p65, 1:500, Nordic Biosite) and adhesion molecules (ICAM-1, 1:500, R&D Systems, UK and VCAM-1, 1:100, Chemicon® International). Photographs of histological sections were taken using Olympus AX70 microscope (Olympus Optical) and analyses were performed with AnalySIS software (Soft Imaging System GmbH).

**Aortic Gene Expression Analyses**

Aortas were collected from PBS perfused 15-month-old mice on Western diet. After removing adventitia, aortas were opened longitudinally and the tissue samples for gene expression analyses were taken from both lesions and intact part and snap frozen in liquid nitrogen. Total RNA was then extracted using TRI® reagent (Sigma, USA). For real-time quantitative RT-PCR, total RNA was reverse transcribed into cDNA using random hexamers (Promega) and M-MuLV reverse transcriptase (MBI Fermentas). Quantitative measurements of gene expression were done using Assays-on-demand gene expression products (Applied Biosystems) with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The specific assays used were Mm00436767_m1 (OPN, osteopontin), Mm00475831_m1 (ALP-2, alkaline phosphatase 2), Mm01340178_m1 (BMP-2, bone morphogenetic protein 2), Mm00432050_m1 (Bax), Mm00446190_m1 (IL-6, interleukin 6), Mm00441242_m1 (MCP-1, monocyte chemotactic protein 1) and Mm00432403_m1 (CD36). Measurements were done as duplicates. The expression levels were normalized to 18S ribosomal RNA (Applied Biosystems).
Statistical Analyses

To evaluate statistical significance, independent samples t-test or Mann-Whitney test were used for the appropriate parameters. For the tolerance tests, ANOVA of repeated measures and Tukey’s test were used. P<0.05 was considered significant. The degree of association was measured by Pearson’s correlation coefficient ($r$). Numerical values for each measurement are shown as mean ± SD or mean ± SEM. All statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad Software).
References


