Hypercholesterolemia Reduces Collateral Artery Growth More Dominantly Than Hyperglycemia or Insulin Resistance in Mice

Vincent van Weel, Margreet de Vries, Peter J. Voshol, Robert E. Verloop, Paul H.C. Eilers, Victor W.M. van Hinsbergh, J. Hajo van Bockel, Paul H.A. Quax

Objective—Collateral artery development (arteriogenesis), a vital compensatory mechanism in patients with arterial obstructive disease, may be deregulated by vascular risk factors, eg, diabetes or hypercholesterolemia. Here, we compared the effects of either disturbed glucose metabolism or disturbed lipid metabolism on arteriogenesis.

Methods and Results—Femoral artery occlusion was performed in streptozotocin(STZ)-treated mice, nonobese diabetic (NOD) mice, and insulin-resistant Ob/Ob mice on regular diet, and APOE3*Leiden mice on different hypercholesterolemic diets. Angiography and laser Doppler perfusion analysis of hindlimbs were performed postoperatively. Surprisingly, angiographic arteriogenesis was not impaired in diabetic and insulin-resistant mice. Perfusion recovery in STZ-treated and Ob/Ob mice was only decreased by 19% and 16%, respectively (P<0.05). Furthermore, perfusion recovery was unchanged between high-glycemic and mild-glycemic NOD mice. Angiographic arteriogenesis in APOE3*Leiden mice, however, was markedly impaired at 7 days and 14 days (P≤0.01). Correspondingly, perfusion recovery was 41% decreased in APOE3*Leiden mice (P<0.05). There was an inverse correlation of perfusion recovery with plasma cholesterol (P=0.02), but not with triglyceride, free fatty acid, glucose, or insulin levels.

Conclusions—Hypercholesterolemia reduces arteriogenesis more dominantly than hyperglycemia or hyperinsulinemia in mice. This suggests that a disturbed lipid metabolism as observed in diabetic patients might be crucial for the impairment of collateral formation. (Arterioscler Thromb Vasc Biol. 2006;26:1383-1390.)

Key Words: arteriogenesis ■ cholesterol ■ collateral circulation ■ diabetes ■ NOD mice ■ peripheral vascular disease

Hyperlipidemia and diabetes mellitus are 2 major risk factors for coronary and peripheral arterial disease, in addition to nicotine abuse, hypertension, and other factors, by increasing the progression of atherosclerosis. Moreover, collateral artery development (arteriogenesis), a vital compensatory mechanism in patients with arterial occlusive disease,3,4 is deregulated by both hyperlipidemia5–8 and diabetes.9,10 Poor arteriogenesis may influence the rate of disease progression and susceptibility for therapeutic intervention, such as direct revascularization techniques, exercise training, or experimental therapies to promote arteriogenesis.11,12 Because both hyperlipidemia and diabetes often coexist in patients with arterial obstructive disease, it is difficult to determine which risk factor plays a predominant role in the impairment of collateral formation.

Moreover, evidence is accumulating that a disturbed lipid metabolism is a crucial determinant of the development of diabetes and its complications, such as accelerated atherosclerosis. For example, disordered fat storage and mobilization, mainly involving triglyceride and free fatty acid metabolism, were implicated in the pathogenesis of insulin resistance and type 2 diabetes.13–18 Furthermore, considerable attention has been drawn to the glycation and/or oxidation of lipoproteins as a reason for accelerated atherosclerosis in type 1 diabetic patients.19,20

In the present study, we wished to compare the effects of either disturbed glucose metabolism or disturbed lipid metabolism on vascular growth. For this, we used a mouse model of hindlimb ischemia that enabled us to study both arteriogenesis and angiogenesis. Arteriogenesis is the development of large conductance vessels, known as collateral arteries, from a pre-existing arteriolar network, which occurs at the level of arterial occlusion, whereas angiogenesis is the formation of small neocapillaries in ischemic tissues more distally.21,22 It is thought that arteriogenesis is more important for restoration of blood flow toward ischemic tissues than angiogenesis.3

We show that hypercholesterolemia reduces arteriogenesis more dominantly than hyperglycemia or insulin resistance.
Diabetic or Insulin-Resistant Mouse Models

Mild Reduction of Collateral Artery Growth in Diabetic or Insulin-Resistant Mouse Models

Parameters of lipid metabolism in plasma

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 WT</th>
<th>STZ</th>
<th>Ob/Ob</th>
<th>APOE3*L+ Chow Diet</th>
<th>APOE3*L+ HFC Diet W</th>
<th>APOE3*L+ HFC Diet N</th>
<th>NOD Mild Glycemia</th>
<th>NOD High Glycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mmol/L</td>
<td>1.91 ± 0.12</td>
<td>1.18 ± 0.23*</td>
<td>3.22 ± 0.30**</td>
<td>2.67 ± 0.42**</td>
<td>12.30 ± 0.45**</td>
<td>36.77 ± 2.20**</td>
<td>1.87 ± 0.14</td>
<td>1.65 ± 0.11</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>0.74 ± 0.08</td>
<td>0.40 ± 0.15*</td>
<td>0.78 ± 0.18</td>
<td>1.67 ± 0.01**</td>
<td>1.53 ± 0.20**</td>
<td>1.70 ± 0.51*</td>
<td>0.99 ± 0.12</td>
<td>1.02 ± 0.16</td>
</tr>
<tr>
<td>FFA, mmol/L</td>
<td>0.55 ± 0.08</td>
<td>0.48 ± 0.09</td>
<td>0.59 ± 0.06</td>
<td>0.40 ± 0.04</td>
<td>1.07 ± 0.08**</td>
<td>0.88 ± 0.20*</td>
<td>0.96 ± 0.14</td>
<td>0.72 ± 0.13</td>
</tr>
</tbody>
</table>

Parameters of glucose metabolism in plasma

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 WT</th>
<th>STZ</th>
<th>Ob/Ob</th>
<th>APOE3*L+ Chow Diet</th>
<th>APOE3*L+ HFC Diet W</th>
<th>APOE3*L+ HFC Diet N</th>
<th>NOD Mild Glycemia</th>
<th>NOD High Glycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>12.23 ± 0.79</td>
<td>24.13 ± 4.28**</td>
<td>12.25 ± 1.53</td>
<td>11.75 ± 0.06</td>
<td>12.21 ± 1.03</td>
<td>13.35 ± 0.73</td>
<td>17.23 ± 1.84**</td>
<td>30.05 ± 0.73**††</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.77 ± 0.12</td>
<td>0.68 ± 0.29</td>
<td>22.90 ± 8.11*</td>
<td>0.83 ± 0.19</td>
<td>0.57 ± 0.17</td>
<td>0.84 ± 0.39</td>
<td>0.31 ± 0.03**</td>
<td>0.10 ± 0.06**††</td>
</tr>
<tr>
<td>Animal weight, g</td>
<td>24.71 ± 0.42</td>
<td>25.53 ± 0.57</td>
<td>40.86 ± 2.34**</td>
<td>22.63 ± 0.30</td>
<td>22.63 ± 0.30</td>
<td>25.87 ± 0.70</td>
<td>29.90 ± 0.84**</td>
<td>28.20 ± 2.40*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

*P<0.05, **P<0.01.
For NOD mice, data were also compared between high- and mild-glycemic mice (††P<0.01).

Materials and Methods

Mice

Experiments were approved by the committee on animal welfare of the Netherlands Organization for Applied Scientific Research (TNO). All animals were male and aged 10 to 20 weeks, except male nonobese diabetic (NOD) mice, which were aged 20 weeks. Numbers of mice per group varied from 3 to 12 (Table 1). Type 1 diabetes models consisted of streptozotocin (STZ)-treated C57BL/6 mice (TNO) and NOD mice (ICR background; Taconic Farms, Ejby, Denmark).2,24 The former mice were rendered diabetic by intraperitoneal injection of 40 mg/kg per day of STZ (Sigma) in citrate buffer, 0.05 mol/L, pH 4.5, for 5 days. Two days after the fifth injection, nonfasting whole-blood glucose levels were monitored. (For description of glucose analysis please see http://atvb.ahajournals.org). Mice with glycemia >10 mmol/L were included in the study, and surgery was performed 7 days later. In NOD mice, whole-blood glucose levels were measured weekly until 50% of the animals developed high glycemia (whole-blood glucose >10 mmol/L). Subsequently, surgery was performed in animals with either mild or high glycemia. At that time, mice were aged 20 weeks. Mice were defined as either being mild glycemic if whole-blood glucose levels were <5 mmol/L and plasma glucose levels were <20 mmol/L, or as being high glycemic if whole-blood glucose levels were >10 mmol/L and plasma glucose levels were >20 mmol/L. For the mild glycemia group, only animals that sustained mild plasma glucose levels, as measured 14 days after surgery, were included. Ob/Ob mice (TNO) were used as a model of insulin resistance.25 (For description of the lipid, glucose, and insulin analysis please see http://atvb.ahajournals.org).

Specific pathogen-free transgenic APOE3*L-Leiden mice were crossbred for >18 generations with C57BL/6 mice (TNO).26 APOE3*L-Leiden mice were allocated randomly to 1 of the 3 experimental diets (please see http://atvb.ahajournals.org).

Induction of Hindlimb Ischemia and Analysis of Collateral Formation

Surgical induction of hindlimb ischemia, as well as analysis of collateral formation by either laser Doppler perfusion imaging or angiography, and capillary formation, were performed as described previously.27,28 For a detailed account of the methodologies used please see http://atvb.ahajournals.org.

Data Analysis

Results are expressed as mean ± SEM. Comparisons between means were performed using 1-way ANOVA test with least significant difference post-hoc analysis. Rentrop scores were compared between groups by cross-classification using the Pearson χ² test. Single and multiple linear regression was used to study relationships. P<0.05 were considered statistically significant. All calculations were performed in SPSS.

Results

Analysis of Arteriogenesis in a Mouse Model of Acute Hindlimb Ischemia

After femoral artery occlusion, a rapid increase of collateral vessel development (arteriogenesis) occurred in the upper limb region of C57BL/6 WT mice. Premature collaterals were angiographically visible within 3 days, and further developed throughout the 28-day observation period (Figure 1A). Filling of distal femoral arteries with contrast medium via collaterals occurred in 17% of mice immediately after femoral artery occlusion (N=6), in 22% of mice at 3d (N=9), in 89% of mice at 7 days (N=9), and in 100% of mice at 14 days and 28 days (N=6 and 9, respectively). Angiographic Rentrop scores for all time points are depicted in Figure 1C. Laser Doppler analysis showed an almost identical time course of recovery of paw perfusion after femoral artery occlusion (n=12) (Figure 1B, 1C). The rapid recovery of paw perfusion was paralleled by only sporadic necrosis of the toes, indicating that the model applied is a transient ischemic model (for necrosis data please see Table I, available online at http://atvb.ahajournals.org).

Plasma Lipid, Glucose, and Insulin Levels

Nonfasting plasma cholesterol, triglyceride (TG), free fatty acid (FFA), glucose, and insulin levels in the various mouse models of hyperlipidemia, diabetes, or insulin resistance at the day of surgery are depicted in Table 1. As expected, plasma lipid levels were markedly increased in APOE3*L-Leiden mice on hypercholesterolemic diet, whereas glucose or insulin levels were increased in diabetic or insulin-resistant mice, respectively.

Mild Reduction of Collateral Artery Growth in Diabetic or Insulin-Resistant Mouse Models

Collateral artery growth was studied in a type 1 diabetes model, namely STZ-treated mice, and in insulin-resistant Ob/Ob mice.
by surgical occlusion of the femoral artery. There was no significant change in angiographic score of collaterals in both STZ-treated and Ob/Ob mice at all time points after surgery as compared with control mice (n=6) (Figure 2A, 2B). Perfusion recovery in STZ-treated and Ob/Ob mice was only decreased with a mean value of 19% and 16%, respectively, from 3 through 28 days after surgery as compared with control mice (P<0.05 at 3 and 14 days, and 3, 14, and 21 days, respectively, n=7) (Figure 2C).

As it was previously reported that ischemia-induced angiogenesis is markedly impaired in NOD mice,9,10 which develop type 1 diabetes by immune attack of their pancreas, we wished to study in more detail the effects of hyperglycemia on collateral formation in NOD mice. Within 20 weeks, 50% of the animals developed marked diabetes with whole-blood glucose levels >10 mmol/L, whereas the other animals remained normoglycemic or mildly glycemic. To study the contribution of hyperglycemia on collateral artery growth, perfusion recovery was analyzed in NOD mice with either mild glycemia or high glycemia (plasma glucose levels 17.2±1.8 mmol/L, n=5, or 30.1±0.7 mmol/L, n=4, respectively). Perfusion recovery was not significantly changed in high-glycemic mice as compared with mild-glycemic mice at all time points after surgery (Figure 2C). However, as compared with C57BL/6 WT mice, both the...
Severely Impaired and Cholesterol-Dependent Collateral Artery Growth in Hyperlipidemic APOE3*Leiden Mice

To determine the effect of hyperlipidemia on collateral formation, we occluded the femoral artery in APOE3*Leiden mice on a hypercholesterolemic diet-N. Angiographic collateral artery growth was significantly reduced in APOE3*Leiden mice on diet-N as compared with C57BL/6 WT mice 7 days after femoral artery occlusion (Rentrop score 1.1 ± 0.3 versus 2.4 ± 0.2, respectively; P = 0.003, n = 7) and 14 days (Rentrop score 2.0 ± 0.37 versus 3.0 ± 0, respectively; P = 0.01, n = 6) (Figure 3A, 3B). Correspondingly, laser Doppler analysis demonstrated a mean decrease of 41% of perfusion recovery from 3 through 28 days after surgery in ischemic hindlimbs of APOE3*Leiden mice on diet-N as compared with control (P < 0.05 at all time points, n = 5) (Figure 3C).

To study whether impairment of collateral artery growth is cholesterol-dependent, we made use of the unique ability to easily control cholesterol levels in APOE3*Leiden mice by modulating the percentage of cholate content in the cholesterol-enriched diet. Mean plasma cholesterol levels were 36.8 ± 2.2, 12.3 ± 0.5, 2.7 ± 0.4, or 1.9 ± 0.1 mmol/L in APOE3*Leiden mice fed either hypercholesterolemic diet-N (cholate 0.5%, cholesterol 1%), hypercholesterolemic diet-W (cholate 0.05%, cholesterol 1%), regular chow diet, or in C57BL/6 WT mice fed regular chow diet. A complete listing of plasma lipid, glucose, and insulin levels is depicted in Table 1. Paw perfusion recovery from 3 to 28 days after arterial occlusion was most severely decreased (mean value, 41%) in APOE3*Leiden mice on diet-N (n = 5), only mildly decreased (mean value of 24%) in APOE3*Leiden mice on diet-W (n = 6), and was not changed in APOE3*Leiden mice on regular diet (n = 3) as compared with C57BL/6 WT mice fed regular diet (n = 8) (Figure 3C). There was a significant inverse correlation between perfusion recovery and plasma cholesterol levels in APOE3*Leiden mice on the different diets from 7 through 28 days after surgery (Figure IIB, available online at http://atvb.ahajournals.org).

To exclude an effect of elevated cholate levels on arteriogenesis, an additional experiment was performed in C57BL/6 mice on either regular chow diet without cholate or hypercholesterolemic diet-N with cholate (N = 7). Plasma cholesterol levels were 2.51 ± 0.32 or 4.57 ± 0.66 mmol/L for C57BL/6 mice on chow diet or diet-N, respectively (P = 0.01). Perfusion recovery was not significantly different between both groups at all time points, indicating that elevated cholic acid levels do not affect arteriogenesis (Figure I, available online at http://atvb.ahajournals.org).

Perfusion recovery was only significantly correlated with plasma cholesterol levels, not with TG, FFA, glucose, or insulin levels, 7 days after femoral artery occlusion, as determined by multiple regression analysis of data derived from all models applied (Table 2; Figure III, available online at http://atvb.ahajournals.org). The 7-day time point was selected because at that time there was a maximum rate of angiographic collateral growth in C57BL/6 mice, as depicted in Figure 1C. Thus, differences in perfusion at that time-point best reflect differences in collateral growth.

No Impairment of Ischemia-Induced Angiogenic Response in Both Hyperlipidemic and Diabetic Mice

Distal to the femoral artery occlusion, in the lower limb, an increased capillary density (angiogenesis) was observed in ischemic as compared with nonischemic calf muscle of C57BL/6 WT mice at 7 days and 14 days (P < 0.03 and 0.004, respectively, n = 7) (Figure 4A, 4B). This was followed by a decrease of capillary number in ischemic limb at 28 days
(n=7). At the latter time point, there was again no significant difference in capillary density between ischemic and nonischemic limb. These data suggest regression of ischemia-induced neovessels.

To study the effect of increased glycemia, insulinemia, or lipidemia on ischemia-induced angiogenesis, we compared capillary density and area per capillary in ischemic calf muscle between the various mouse groups at 14 days after femoral artery occlusion, when capillary density reached a maximum in C57BL/6 WT mice. Ischemic/nonischemic capillary density ratio was unchanged between all groups tested at 14 days, indicating a similar angiogenic response (n=4) (Figure 4C, 4D). Capillaries were, however, significantly enlarged in ischemic muscle of APOE3*Leiden mice as compared with C57BL/6 WT mice (P<0.05) (Figure 4C, 4E).

Discussion

In the present study, it was demonstrated that arteriogenesis is markedly impaired by hypercholesterolemia, but only mildly impaired by hyperglycemia or insulin resistance. Moreover, we show an inverse correlation between plasma cholesterol levels and the ability to develop collateral arteries.

Recently, evidence is building that a disturbed lipid metabolism is associated with both the development of diabetes and its complications, particularly accelerated atherosclerosis.13–20 It is therefore tempting to hypothesize that a disturbed lipid metabolism also plays a crucial role in the impairment of arteriogenesis, another important complication that is observed in diabetic patients.9 In the present study, we found evidence for this, by showing that a disturbed lipid metabolism is more crucial for impairment of arteriogenesis than a disturbed glucose metabolism.

First, we studied the effect of hyperglycemia or insulin resistance on arteriogenesis in mice. Collateral formation was angiographically unaltered in STZ-induced type-1 diabetic mice with high glucose levels, and in insulin-resistant Ob/Ob mice. We used Ob/Ob mice with normalized glucose levels, as reported,29 whereas insulin levels were profoundly elevated, allowing us to restrictedly study the role of hyperinsulinemia on arteriogenesis.

These findings were somewhat surprising because Rivard et al previously reported that ischemia-induced angiogenesis is retarded in a well-established model of type 1 diabetes, namely NOD mice.10,23,24 It should be realized, however, that in this study the whole femoral and saphenous artery, as well as all side branches, were excised, whereas here the femoral artery was occluded proximally over a short distance. As only with the latter technique the pre-existing collateral network remains connected to distal arteries, allowing arteriogenesis to occur, we wished to repeat the experiment in NOD mice using our modified less extreme surgical procedure. Moreover, Rivard et al performed their experiments in hyperglycemic NOD mice using C57BL/6 mice as control. We here compared high-glycemic NOD mice with mild-glycemic NOD mice of the same ICR background in addition to C57BL/6 WT mice. Paw perfusion recovery after femoral artery occlusion was similar between high-glycemic NOD mice and their mild-glycemic littermates. However, when NOD mice were compared with C57BL/6 mice, perfusion ratios were markedly decreased for both high-glycemic and mild-glycemic NOD mice, which is comparable to previously reported data.10 These findings suggest that strain-dependent factors contribute to impairment of collateral artery growth, independent of glucose levels. For example, T cell–mediated immunity differs between NOD mice and C57BL/6 mice.30,31 T cells are thought to play a crucial role in arteriogenesis.32 Furthermore, because NOD mice have normal plasma cholesterol levels, it is unlikely that cholesterol or its metabolites caused reduced collateral formation in NOD mice. To avoid any possible strain-dependent effects on collateral formation, we evaluated the effects of type 1 diabetes in another mouse model of diabetes than NOD mice, namely STZ-treated C57BL/6 mice, and compared these with their nontreated C57BL/6 littermates, as described. Together, these data suggest that other factors than hyperglycemia might explain the impaired arteriogenesis in diabetic patients. Nevertheless, it should be pointed out that the relatively short duration of diabetes in our mouse models as opposed to chronically disturbed glucose metabolism in diabetic patients may be a limitation of the study. We cannot exclude that prolonged exposure of the vessel wall to elevated glucose levels may lead to impaired collateral formation due to changed glycation pattern of proteins, as proposed.19,20,33

In hyperlipidemic APOE3*Leiden mice fed a high-fat diet, a profound retardation of collateral formation was found after femoral artery occlusion. Impairment of collateral growth by hyperlipidemia was previously shown in APOE−/− mice.4 Here, APOE3*Leiden mice were used because the lipoprotein...
profile in these mice closely resembles that of humans. In addition, plasma cholesterol levels could easily be modulated in these mice by changing diets. Subsequent changes in cholesterol levels in the APOE3*Leiden mice showed a strong inverse correlation with perfusion recovery after femoral artery occlusion. Because the increase of plasma cholesterol levels in high-fat diet-fed APOE3*Leiden mice is mainly observed in the VLDL and LDL fractions, we hypothesize that alterations of these fractions may play a crucial role in the disturbance of collateral formation. It should be noted that APOE3*Leiden mice are known to develop insulin resistance when fed a diet with very high fat percentage (23%) for 20 weeks or more. In the present study, however, APO3E*Leiden mice were fed diets with a lower fat percentage (15%) for only 4 weeks, persisting for another 4 weeks during the experiment. Consequently, there were no signs of insulin resistance present in these mice, such as elevated glucose or insulin levels (Table 1), allowing us to study the effect of lipid metabolism independent of glucose metabolism.

The exact cellular mechanisms mediating the adverse effects of lipids on collateral artery formation remain to be determined. One previously proposed mechanism is that endothelial cell motility is hampered by (lipid components of) oxidized LDL. The same lipids that inhibit movement of endothelial cells stimulate movement of monocytes, T lymphocytes, and smooth muscle cells. The mentioned inflammatory cells play a crucial role in the development of atherosclerosis. Recently, it was proposed that inflammatory responses involved in atherosclerotic plaque progression also contribute to collateral formation. Therefore, it may well be possible that a disturbed lipid metabolism impairs collateral formation by modulating the function of inflammatory cells, such as monocytes/macrophages, T lymphocytes, or their receptors, which have been implicated in arteriogenesis. This may, for instance, lead to disturbed arteriogenic cytokine profiles produced by these cells in the collateral vessel wall.

In line with our data, it was recently shown that cholesterol reduction with statins improves walking distance in patients with peripheral artery disease. From this, it could be speculated that statins may improve blood vessel formation by reducing the high cholesterol levels.

In addition, inactivation of nitric oxide (NO) by reactive oxygen species (ROS) production in the vascular wall seems to occur in pathological conditions such as hypertension, hypercholesterolemia, diabetes, and cigarette smoking. Endothelial NO synthesis has been implicated in ischemia-induced neovascularization. Therefore, pathological ROS production and thereby inactivation of NO may also contribute to delineating a difference in arteriogenesis between hypercholesterolemic and hyperglycemic or hyperinsulinemic conditions.

Finally, we show that capillary formation in tissues distal to the arterial occlusion is selectively increased in hyperlipidemic mice and unaffected in diabetic mice as compared with wild-type mice, whereas collateral artery development in hyperlipidemic mice was markedly impaired. We propose that hyperlipidemic mice developed more profound ischemia.
in distal tissues, and consequently ischemia-induced vessel growth, because of the severely impaired collateral inflow. Together, these data underscore that there is a dissociation between angiogenesis and arteriogenesis, as proposed previously.21,22

In conclusion, impairment of arteriogenesis is more associated with hyperlipidemia than hyperglycemia or hyperinsulinemia, and it is cholesterol-dependent. Therefore, a disturbed lipid profile as observed in many diabetic patients might be crucial for the impairment of collateral formation in these patients. Moreover, our findings comply with the new guidelines from the American College of Physicians that most patients with type 2 diabetes should be treated with lipid-lowering medication to help prevent cardiovascular mortality and morbidity, regardless of cholesterol levels.45

Acknowledgments

This study was sponsored by the TNO-LUMC-VUMC tripartite angiogenesis program, the NHLS Molecular Cardiology Program (M93.001), the STW/DTPE Dutch program on Tissue Engineering (VTG.6747), and the European Vascular Genomics Network (L3HM-CT-2003-503254). We acknowledge Kees van Leuven (TNO, Leiden) for his technical assistance.

References


Hypercholesterolemia Reduces Collateral Artery Growth More Dominantly Than Hyperglycemia or Insulin Resistance in Mice

Vincent van Weel, Margreet de Vries, Peter J. Voshol, Robert E. Verloop, Paul H.C. Eilers, Victor W.M. van Hinsbergh, J. Hajo van Bockel and Paul H.A. Quax

Arterioscler Thromb Vasc Biol. 2006;26:1383-1390; originally published online March 30, 2006;
doi: 10.1161/01.ATV.0000219234.78165.85

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/6/1383

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/04/02/01.ATV.0000219234.78165.85.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Extended Materials and Methods

Diets

APOE3*Leiden mice were fed either a chow-diet, a high-fat cholesterol-enriched diet containing 0.5% cholate to improve intestinal cholesterol uptake and suppress bile acid synthesis, both leading to increased plasma cholesterol levels (Diet N: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.5%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn-oil 1%, cellulose 5.1%, mineral mixture 5.1%) or a cholesterol-enriched high-fat diet containing 0.05% cholate (Diet W: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.05%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn-oil 1%, cellulose 4.7%, mineral mixture 5.1%) 4 weeks prior to surgery and continued after surgery. All diabetic mice were fed a regular chow-diet.

Surgical induction of hindlimb ischemia

Mice were anesthetized with a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen) intraperitoneally before surgery. Ischemia of the left hindlimb was induced by coagulation of the left femoral artery proximal to the bifurcation of superficial and deep femoral artery.
Lipids, glucose and insulin analysis

Blood samples were taken under general anesthesia at the time of surgery following free feeding between 9h00 and 11h00 am. Total plasma cholesterol (Roche Diagnostic), triglyceride (TG; Roche Diagnostics) and free fatty acid (FFA; WAKO) concentrations were measured enzymatically using commercially available kits. Whole-blood or plasma glucose levels were measured using the Freestyle™ glucometer (Ypsomed) or a commercially available kit (INstruchemie), respectively. Plasma insulin levels were estimated by the ELISA method using a commercially available kit (Mouse insulin, Mercordia AB).

Angiography

To study collateral formation, post-mortem angiography of both hindlimbs was performed using polyacrylamide-bismuth contrast (Sigma) at various time points after femoral artery occlusion, as described.¹ Grading of collateral filling was performed in a single blinded fashion and was based on the Rentrop Score. Grading was as follows: 0=no filling of collaterals, 1=filling of collaterals only, 2=partial filling of distal femoral artery, 3=complete filling of distal femoral artery.

Laser Doppler Perfusion Imaging

Perfusion analysis of both paws was performed at baseline, immediately after surgery, and serially over 4 weeks, using Laser-Doppler Perfusion Imaging (LDPI) (Moor Instruments), as described.² Perfusion is expressed as a ratio of left (ischemic) to right (non-ischemic) paw.
Analysis of capillary density

To study formation of capillaries in ischemic muscle, endothelial immunostaining was performed on 5 µm-thick paraffin-embedded sections of bilateral gastrocnemius muscles. For this, sections were re-hydrated and pre-incubated with 100% methanol/0.3% H₂O₂ in phosphate buffered saline (PBS) for 20 minutes to abolish endogenous peroxidase activity, followed by incubation with 0.1% trypsin (Fluka BioChemica) in PBS for 30 minutes at 37°C for antigen unmasking. Sections were incubated overnight at 4°C with a monoclonal rat-anti-mouse antibody, which recognizes CD31 (BD Pharmingen) at a 1:200 dilution. As secondary antibody a biotinylated goat-anti-rat antibody (BD Pharmingen) was used at a 1:300 dilution. After incubation with an avidin-biotin complex (ABC, Dako) the immunohistochemical reaction was enhanced by tyramine amplification, as described. After a second incubation with ABC, antibodies were visualized with the Novared substrate kit (Vector Laboratories) and sections were counterstained by Mayer’s hematoxylin. One section per limb was analysed and consisted of transversely cut muscle tissue derived from the anatomic middle part of the gastrocnemius muscle between proximal and distal tendon. Capillary density and area per capillary were quantified from a minimum of 10 photographed images per section using image analysis (Qwin, Leica).
Figure I. No effect of high cholate levels on perfusion recovery in C57BL/6 mice.
Figure II

A. No significant correlation between perfusion recovery and glucose levels in NOD mice at all time points after femoral artery occlusion (R=0.316, p=0.407, Day 14).

B. Significant inverse correlation between perfusion recovery and plasma cholesterol levels in APOE3*Leiden mice on different diets from 7 through 28 days after femoral artery occlusion (R=-0.699, p=0.008, Day 14).
Figure III

Multiple linear regression analysis of ischemic/non-ischemic paw perfusion ratio versus plasma cholesterol, triglyceride, free fatty acids, glucose or insulin levels 7 days after femoral artery occlusion in the various hypercholesterolemic and diabetic mouse models. Scatter plots with mean regression prediction line and 95% confidence interval.
Table I. Necrosis data for the various models as observed 7 days after femoral artery occlusion.

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 WT</th>
<th>STZ</th>
<th>Ob/Ob</th>
<th>APOE3*L+ Chow diet</th>
<th>APOE3*L+ HFC diet W</th>
<th>APOE3*L+ HFC diet N</th>
<th>NOD mild glycemia</th>
<th>NOD high glycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice per group</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Number of mice with necrosis of toes</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Number of toes affected</td>
<td>2x2</td>
<td>1x4</td>
<td>1x2 and 2x1</td>
<td>0</td>
<td>2x1</td>
<td>1x3 and 1x1</td>
<td>1x2 and 1x1</td>
<td>1x4 and 1x1</td>
</tr>
</tbody>
</table>
