Growing blood vessels to treat limb ischemia : studie in mice and man

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Chapter 5

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

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Abstract

Objective: Collateral artery development (arteriogenesis), a vital compensatory mechanism in patients with arterial obstructive disease, may be deregulated by vascular risk factors, e.g. 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, non-obese 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 hind limbs 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 ($p\leq0.01$). 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.

Introduction

Hyperlipidemia and diabetes mellitus are two major risk factors for coronary and peripheral arterial disease, in addition to nicotine abuse, hypertension and other factors, by increasing the progression of atherosclerosis.[1] Moreover, collateral artery development (arteriogenesis), a vital compensatory mechanism in patients with arterial occlusive disease[2;3], is deregulated by both hyperlipidemia[4-8] 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] Since 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 hind limb 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 neo-capillaries in ischemic tissues more distally.[21;22] It is thought that arteriogenesis is more important for restoration of blood flow towards ischemic tissues than angiogenesis.[3]

We show that hypercholesterolemia reduces arteriogenesis more dominantly than hyperglycemia or insulin resistance.

Materials and methods

Mice

All experiments were approved by the committee on animal welfare of the Netherlands Organization for Applied Scientific Research (TNO). Male mice were used, aged 10-12 weeks. Male non-obese-diabetic (NOD) mice were aged 20 weeks.

Numbers of mice per group varied from 3 to 12 (see also Table 1). Type-1 diabetes models consisted of streptozotocin(STZ)-treated C57BL/6 mice (TNO) and NOD mice (ICR background) (Taconic Farms).[23;24] The former mice were rendered diabetic by intraperitoneal injection of 40mg/kg/day of STZ (Sigma) in citrate buffer, 0.05 M, pH 4.5, for 5 days. Two days after the fifth injection, non-fasting whole-blood glucose levels were monitored. Mice with glycemia >10mM 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>10mM). 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 mild glycemic if whole blood glucose levels were below 5mM and plasma glucose levels were below 20mM, or as high glycemic if whole blood glucose levels were above 10mM and plasma glucose levels were above 20mM. 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] Specific-Pathogen-Free Transgenic APOE3*Leiden mice were crossbred for >18 generations with C57BL/6 mice (TNO).[26] APOE3*Leiden mice were allocated randomly to one of the 3 experimental diets, as described below.

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 FreestyleTM 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).

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 high-fat cholesterol-enriched 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.

Induction of hind limb Ischemia and analysis of collateral formation

Surgical induction of hind limb 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];
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 hind limb was induced by coagulation of the left femoral artery proximal to the bifurcation of superficial and deep femoral artery.
To study collateral formation, post-mortem angiography of both hind limbs was performed using polyacrylamide-bismuth contrast (Sigma) at various time points after femoral artery occlusion. 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. 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). Perfusion is expressed as a ratio of left (ischemic) to right (non-ischemic) paw.
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% H_2O_2 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. 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 analyzed 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).

Statistical analysis

Results are expressed as mean±SEM. Comparisons between means were performed using one-way ANOVA test with LSD post-hoc analysis. Rentrop scores were compared between groups by cross-classification, using the Pearson chi-square test. Single and multiple linear regression were used to study relationships. P-

values <0.05 were considered statistically significant. All calculations were performed in SPSS.

Results

Analysis of arteriogenesis in a mouse model of acute hind limb ischemia

After femoral artery occlusion, a rapid increase of collateral vessel development (arteriogenesis) occurred in the upper limb region of C57BL/6 WT mice. Pre-mature collaterals were angiographically visible within 3 days, and further developed throughout the 28 days 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 3 days (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,C). 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 (Table 1).

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

Table 1 Necrosis data for the various models as observed 7 days after femoral artery occlusion

Plasma lipid, glucose and insulin levels

Non-fasting 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 2. As expected, plasma lipid levels were markedly increased in APOE3*Leiden mice on hypercholesterolemic diet, whereas glucose or insulin levels were increased in diabetic or insulin-resistant mice, respectively.

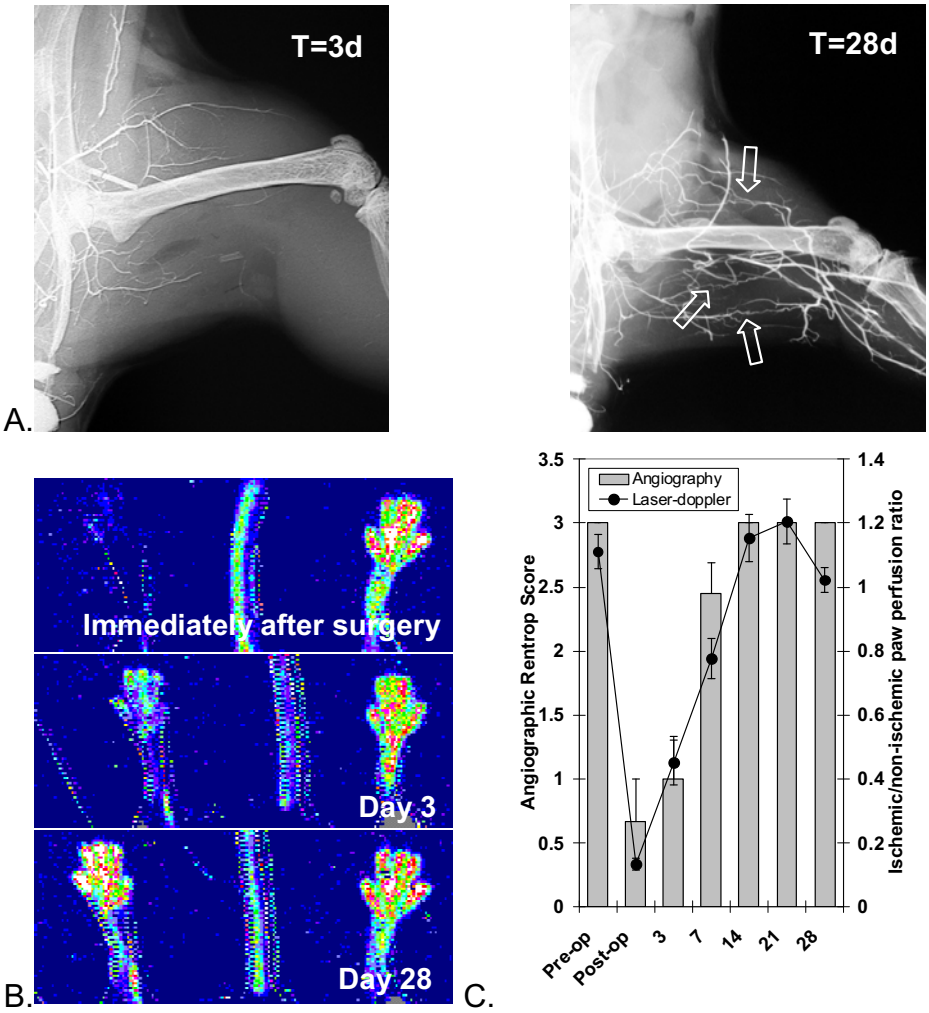


Figure 1 A Representative angiographies of upper hind limb 3 days and 28 days after occlusion in C57BL/6 mice. Well-developed collateral arteries were visible at 28 days (arrows). **B** Perfusion images of both paws after unilateral femoral artery occlusion (left paw in picture) in C57BL/6 mice. **C** Quantification of both angiographic collateral arteries (n=6) and perfusion recovery (n=8), expressed as Rentrop score and ischemic/non-ischemic paw perfusion ratio, respectively.

	C57BL/6 WT	STZ	Ob/Ob	APOE3*L+ Chow diet	APOE3*L+ HFC diet W	APOE3*L+ HFC diet N	NOD Mild glycemia	NOD High glycemia
Parameters of lipid metabolism in plasma								
Cholesterol (mM)	1.91±0.12	1.18±0.23*	3.22±0.30**	2.67±0.42**	12.30±0.45**	36.77±2.20**	1.87±0.14	1.65±0.11
TG (mM)	0.74±0.08	0.40±0.15*	0.78±0.18	1.67±0.01**	1.53±0.20**	1.70±0.51*	0.99±0.12	1.02±0.16
FFA (mM)	0.55±0.08	0.48±0.09	0.59±0.06	0.40±0.04	1.07±0.08**	0.88±0.20*	0.96±0.14*	0.72±0.13
Parameters of glucose metabolism in plasma								
Glucose (mM)	12.23±0.79	24.13±4.28**	12.25±1.53	11.75±0.06	12.21 ± 1.03	13.35±0.73	17.23±1.84**	30.05±0.73**††
Insulin (ng/ml)	0.77±0.12	0.68±0.29	22.90±8.11*	0.83±0.19	0.57±0.17	0.84±0.39	0.31±0.03**	0.10±0.06**††
Animal weight (g)	24.71±0.42	25.53±0.57	40.86±2.34**	22.63±0.30	22.63±0.30	25.87±0.70	29.90±0.84**	28.20±2.40*
N per group	8	7	7	3	6	5	5	4

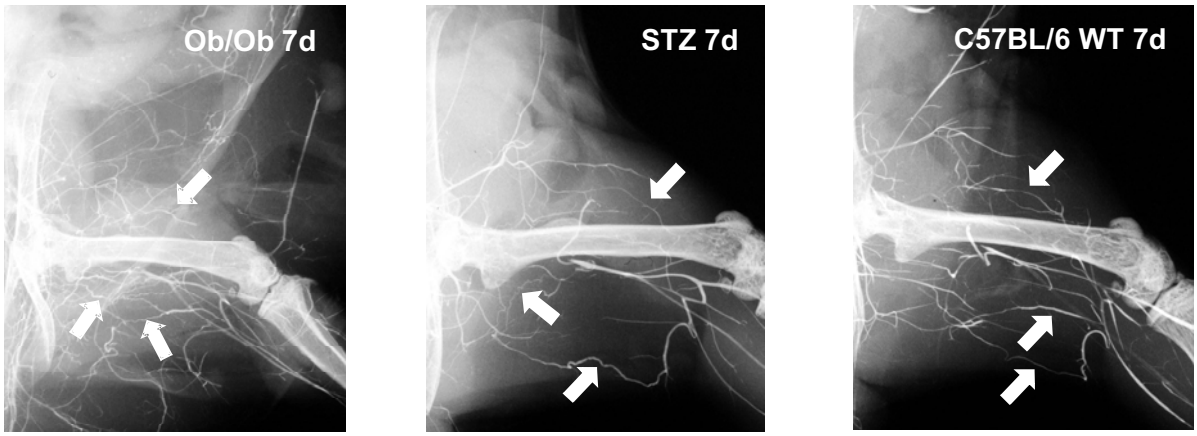
Table 2 Non-fasting plasma cholesterol, triglyceride (TG), free fatty acid (FFA), glucose and insulin levels and animal weight at the day of surgery in mice with diabetes, insulin resistance or hyperlipidemia as compared with C57BL/6 WT mice. Data are expressed as mean±SEM, *p<0.05, **p<0.01. For NOD mice, data were also compared between high- and mild-glycemic mice (††p<0.01).

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 streptozotocin (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,B).
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 non-obese diabetic (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 mM whereas the other animals remained normoglycemic or mild-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±1.8mM, n=5, or 30.1±0.7mM, 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 mild- and high-glycemic NOD mice showed a significantly decreased perfusion recovery by more than 35% 14 days after femoral artery occlusion ($p<0.01$), persisting at 21 days ($p<0.05$). In addition, there was no significant correlation between perfusion recovery and glucose levels in NOD mice at all time-points after surgery (Figure 2D).

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,B). Correspondingly, Laser-Doppler analysis demonstrated a mean decrease of 41% of perfusion recovery from 3 through 28 days after surgery in ischemic hind limbs of APOE3*Leiden mice on diet N as compared with control ($p<0.05$ at all time points, n=5) (Figure 3C).



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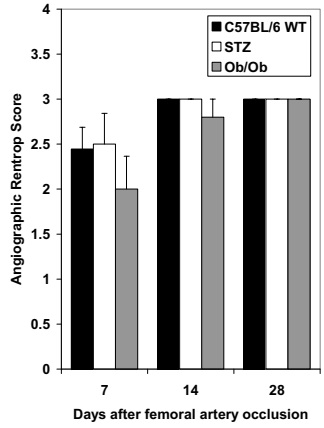

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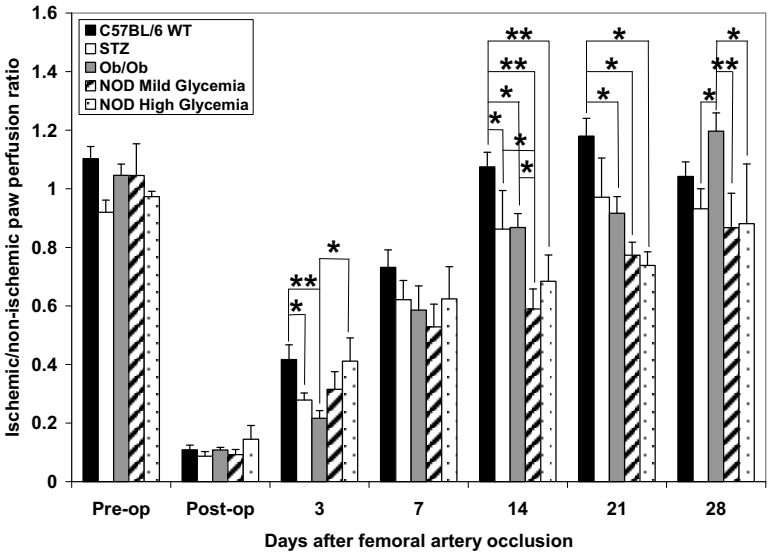

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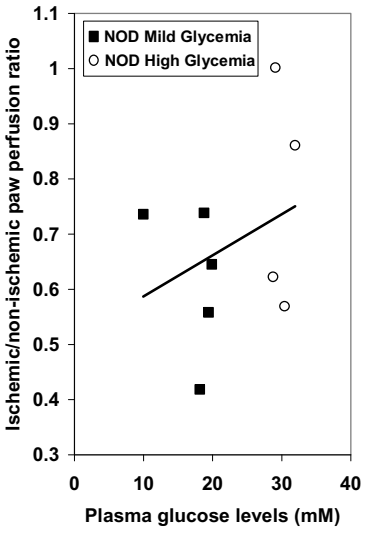

D.

Figure 2 A Representative angiographies of upper hind limb 7 days after surgery in diabetic and insulin-resistant mice. Angiographic collateral formation in STZ-treated C57BL/6 and Ob/Ob mice was comparable to control C57BL/6 WT mice (arrows= collateral arteries). **B** Quantification of angiographic collateral arteries (Rentrop score) (n=6). **C** Ischemic/non-ischemic paw perfusion ratios. Perfusion recovery was mildly decreased in both STZ-treated C57BL/6 and Ob/Ob mice as compared with control C57BL/6 WT mice (n=7). Perfusion recovery was unchanged between high-glycemic and mild-glycemic NOD mice (n=4 and 5 respectively). As compared with C57BL/6 WT mice, however, both mild- and high-glycemic NOD mice showed a decreased perfusion recovery (*p<0.05, **p<0.01). **D** 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).

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±0.1mM in APOE3*Leiden mice fed on 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 on regular chow diet. A complete listing of plasma lipid, glucose and insulin levels is depicted in Table 2. Paw perfusion recovery from 3 through 28 days after femoral artery 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 not changed in APOE3*Leiden mice on regular diet (n=3) as compared with C57BL/6 WT mice fed on 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 3D).

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±0.66mM 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 4).

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 3). The 7-day time-point was selected since 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.

				95% Confidence Interval of Slope	
Perfusion ratio versus:	Pearson Correlation	P-value (2-tailed)	Slope	Lower Bound	Upper Bound
Cholesterol	-0.784*	0.021	–0.007	–0.013	–0.002
Triglyceride	-0.552	0.156	–0.132	–0.332	0.067
Free Fatty Acids	-0.476	0.233	–0.226	–0.643	0.191
Glucose	0.097	0.819	0.002	–0.015	0.018
Insulin	-0.005	0.991	-7.31×10^{-5}	–0.015	0.014

Table 3 Multiple linear regression analysis of ischemic/non-ischemic paw perfusion ratio versus plasma cholesterol, TG, FFA, glucose or insulin levels 7 days after surgery in various hypercholesterolemic and diabetic mouse models. Pearson correlations and slopes with 95% confidence intervals.

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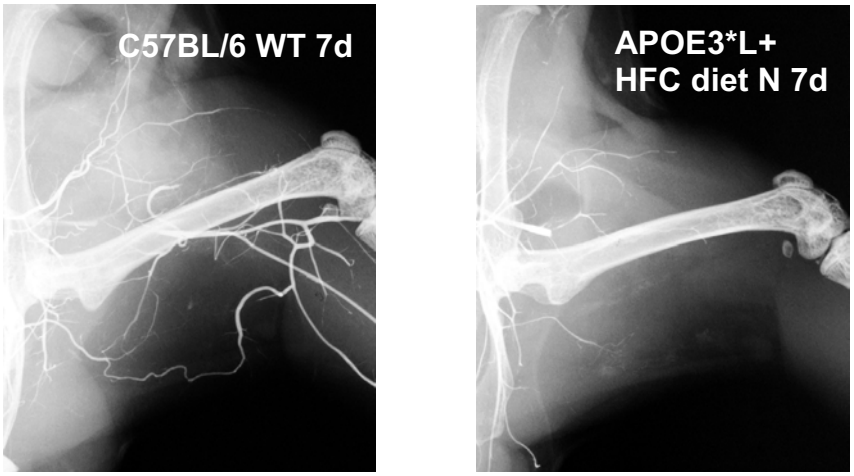

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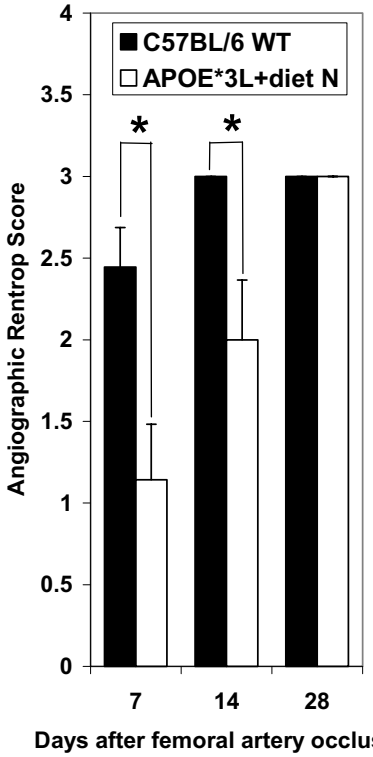

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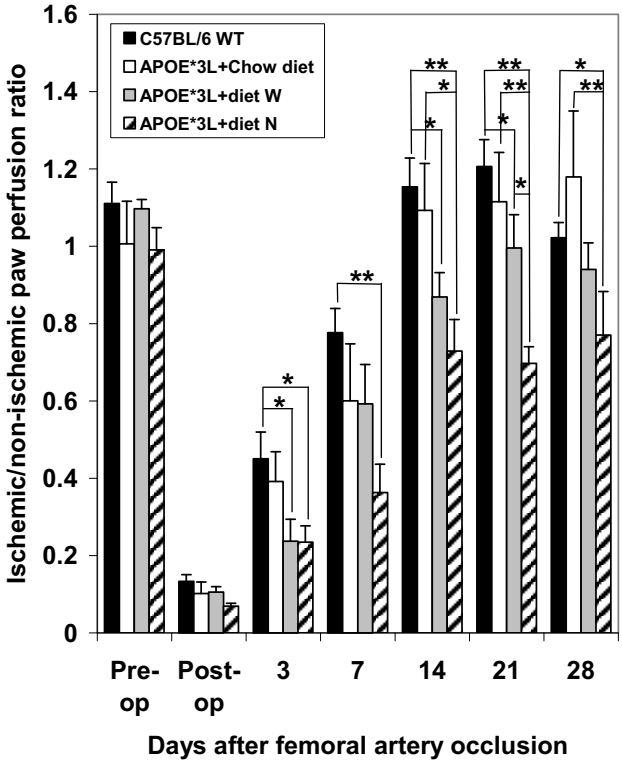

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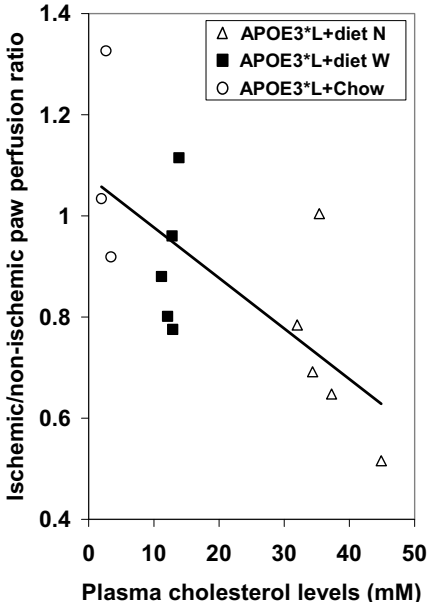


Figure 3 A Representative angiographies of upper hind limb 7 days after surgery. Collateral arteries were absent in APOE3*Leiden mice on hypercholesterolemic diet, whereas many collaterals were visible in C57BL/6 WT mice on regular diet. **B** Quantification of angiographic collateral arteries (Rentrop score) (*p<0.05, **p<0.01, n=5-7). **C** Ischemic/non-ischemic paw perfusion ratios in APOE3*Leiden mice fed on either hypercholesterolemic diet N, hypercholesterolemic diet W or regular chow diet as compared with C57BL/6 WT mice fed on regular chow diet. Perfusion recovery in ischemic paws was most severely hampered in APOE3*Leiden mice on diet N (*p<0.05, **p<0.01, n=5). **D** 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).

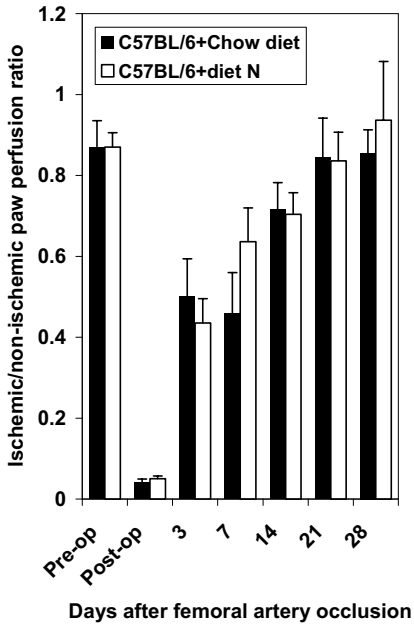


Figure 4 No effect of high cholate levels on perfusion recovery in C57BL/6 mice.

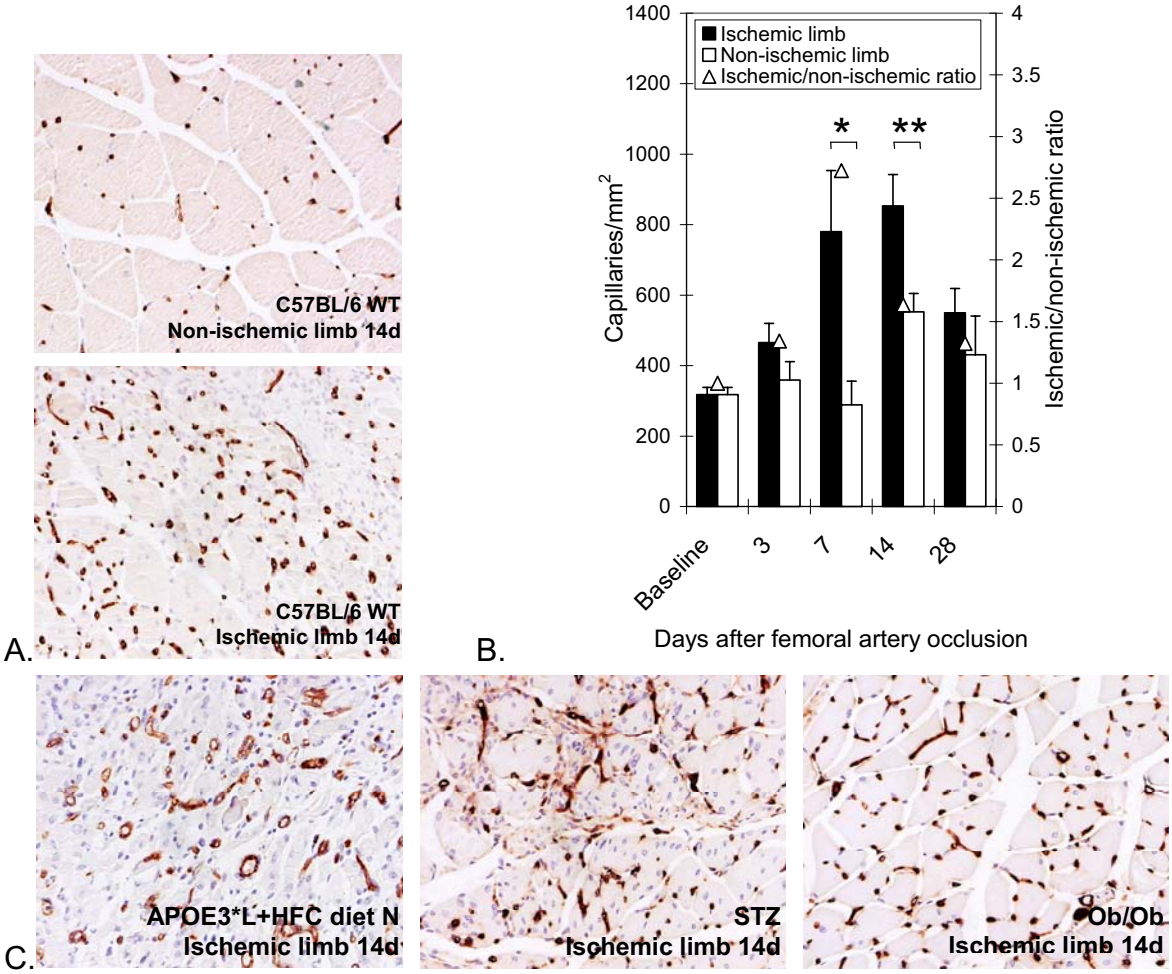
No impairment of ischemia-induced angiogenic response in both hyperlipidemic and diabetic mice

Distal to the femoral artery occlusion, in the lower limb, an increased capillary density (angiogenesis) was observed in ischemic as compared to non-ischemic calf muscle of C57BL/6 WT mice at 7 days and 14 days ($p=0.03$ and 0.004, respectively, $n=7$) (Figure 5A+B). 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 non-ischemic limb. These data suggest regression of ischemia-induced neo-vessels.

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/non-ischemic capillary density ratio was unchanged between all groups tested at 14 days, indicating a similar angiogenic response ($n=4$) (Figure 5C,D). Capillaries were, however, significantly enlarged in ischemic muscle of APOE3*Leiden mice as compared with C57BL/6 WT mice ($p<0.05$) (Figure 5C,E).

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.



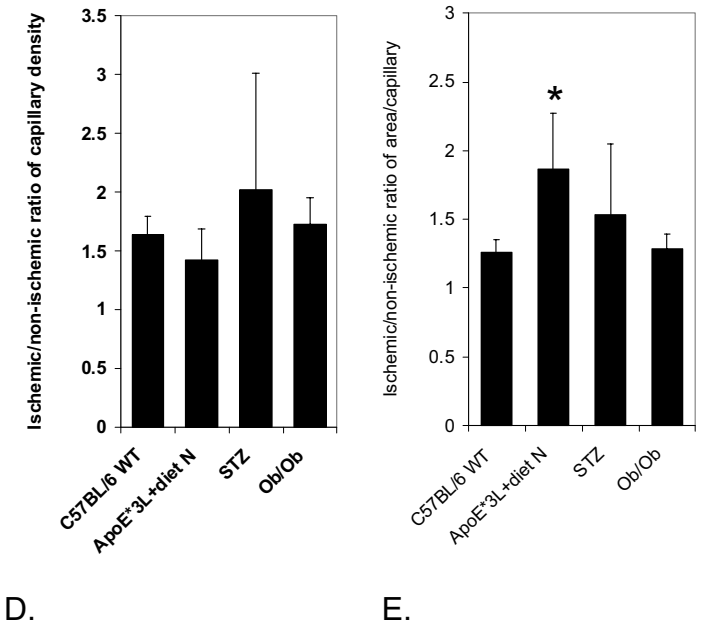


Figure 5 A Representative photomicrographs of anti-CD31-stained gastrocnemius muscle sections derived from ischemic or non-ischemic limb in C57BL/6 mice. (x100). **B** Quantification of CD31-stained sections, including ratios of capillary density between ischemic and non-ischemic limbs (*p<0.05, **p<0.01, n=7). Baseline= non-operated mice. **C** Representative photomicrographs of anti-CD31 staining of ischemic gastrocnemius muscle sections 14 days after surgery in APOE3*Leiden mice on diet N, STZ-treated diabetic C57BL/6 mice or Ob/Ob mice. (x100). **D** Quantification of CD31-stained capillaries showed no difference in capillary density between the various mice (expressed as ischemic/non-ischemic limb capillary density ratio) (n=4). **E** Capillaries were significantly enlarged in ischemic muscle of APOE3*Leiden mice as compared with C57BL/6 WT mice (*p<0.05).

Recently, evidence is building up 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 since 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 its non-treated C57BL/6 littermates, as described above. 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 on 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.[26] 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. Since the increase of plasma cholesterol levels in high-fat diet-fed APOE3*Leiden mice is mainly observed in the very low density lipoprotein (VLDL)- and low density protein (LDL)-sized fractions[26], 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.[34] In the present study, however, APO3E*Leiden mice were fed diets of 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 2), 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 low density lipoprotein.[35;36] The same lipids that inhibit movement of endothelial cells, stimulate movement of monocytes, T-lymphocytes, and smooth muscle cells.[37;38] The mentioned inflammatory cells play a crucial role in the development of atherosclerosis.[39] Recently, it was proposed that inflammatory responses involved in atherosclerotic plaque progression also contribute to collateral formation.[40] 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.[32;41;42] 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 PAD.[6;7] 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.[43] Endothelial NO synthesis has been implicated in ischemia-induced neovascularization.[44]

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 to 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, due to 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 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]

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