

Paradoxical reduction of atherosclerosis in apoE-deficient mice with obesity-related type 2 diabetes

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Abstract

Objective: The effect of obesity and insulin resistance on the development of atherosclerosis was evaluated in apoE-deficient (ApoE^{-/-}) mice. A previously described obesity model, in which the hypothalamic satiety center can be destroyed by a single gold thioglucose (GTG) injection, was used. To evaluate the effect of starvation on atherosclerosis ApoE^{-/-} mice were food-restricted with 25% less chow than ad libitum-fed control mice. **Methods:** Sixty-eight ApoE^{-/-} mice were allocated into a control group ($n=20$), a GTG-injected group ($n=28$), and a food-restricted group ($n=20$). The control and food-restricted mice were injected with saline instead of GTG. The control and GTG-injected mice had free access to food, and all mice had free access to water during the study period. **Results:** After 4 months, the GTG-injected mice were significantly overweight (mean body weight (g): 33 ± 2.11 vs. 23 ± 0.24 and 17 ± 0.31 in control and food-restricted mice, respectively), obese, hypertriglyceridemic, insulin-resistant, hyperinsulinemic (mean plasma insulin (ng/ml): 2.45 and 0.43 in obese and control mice, respectively), and hyperglycemic (mean plasma glucose (mmol/l): 11.03 and 7.80 in obese and control mice, respectively). Unexpectedly, these obese and diabetic mice developed significantly less atherosclerosis compared with lean non-diabetic control mice. Food-restricted mice also developed less atherosclerosis compared to control mice. **Conclusions:** These findings may question the usefulness of mouse models in studying the relation of obesity-related type 2 diabetes to atherosclerosis and also the relevance of results obtained in apoE^{-/-} mice with reduced weight gain during intervention.

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1. Introduction

Type 2 diabetes is characterized by obesity, insulin resistance; hyperinsulinemia, hyperglycemia and dyslipidemia; in combination with essential hypertension also referred as the metabolic syndrome. Type 2 diabetes constitutes an increasing health problem world wide, mainly because of the increasing prevalence of obesity and reduced physical activity [1]. Myocardial infarction, stroke and peripheral vascular disease cause 80% of all diabetic mortality with coronary artery disease as the most frequent cause of death [1]. The overall risk of cardiovascular disease is increased two to four times in patients with type

2 diabetes [2]. Even after adjusting for hypertension and hypercholesterolemia, diabetes in itself remains as an independent risk factor for the development of cardiovascular disease [3,4]. The specific interaction between type 2 diabetes and atherothrombotic disease is, however, poorly understood, partly because of the lack of appropriate animal models.

Therefore, the aim of the present study was to create an animal model in which obesity-induced type 2 diabetes and atherosclerosis coexist in such a way that the effect of diabetes and its metabolic components on atherosclerosis could be examined. On standard chow diet, the hypercholesterolemic apoE^{-/-} mouse develops advanced atherosclerosis spontaneously [5]. The wild-type counter-

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part, the C57BL mouse, becomes obese and insulin-resistant by a single GTG injection, which destroys the hypothalamic neurons regulating food intake and energy expenditure [6]. Therefore, an animal model of innate susceptibility to atherosclerosis with acquired type 2 diabetes superimposed might be created in apoE^{-/-} mice by a single GTG injection.

2. Methods

2.1. Mice, GTG and food restriction

A total of 70 female apoE^{-/-} mice, backcrossed for 10 generations on the C57BL/6J background (M&B, Ry, Denmark), were fed regular pelleted chow (Altromin 1324) with a fat content of 5% (w/w) throughout the study. At 8 weeks of age, the mice were injected i.p. with 0.5 mg GTG (Sigma–Aldrich, Vallensbaek, Denmark) per g body weight ($n=30$) or saline ($n=40$). Two died shortly after GTG-injection, leaving 28 mice in the GTG group. The mice receiving saline were allocated into a control group ($n=20$), and a food-restricted group ($n=20$) that was given 25% less food than that eaten by control mice. Control and GTG-injected mice had free access to food, and all mice had free access to water. The mice were killed 4 months later at the age of 6 months. The mice were maintained five per cage in a temperature-controlled (21 °C) facility with a strict 12-h light/dark cycle. Body weight and food consumption were measured throughout the study. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the mice were housed and cared for according to national guidelines, and the National Animal Ethics Committee approved all the procedures.

2.2. Plasma insulin and glucose

Three weeks before the end of the study, 5-h fasting blood samples (100 μ l, obtained between 10:00 and 12:00 h) were drawn from the retro orbital venous plexus of non-anesthetized mice into chilled tubes containing heparin/aprotinin (Løvens Kemiske Fabrik, Ballerup, Denmark). The blood was centrifuged, and plasma was stored immediately at -20 °C. Glucose was determined by the glucose-oxidase method (GOD/PAP, Boehringer-Mannheim, Mannheim, Germany). Insulin was measured using a sensitive rat insulin kit (Linco Res., MO, USA).

2.3. Insulin sensitivity test

One week before the end of the study, an intraperitoneal insulin sensitivity test (IST) was performed in non-anesthetized mice fasted for 18 h. Blood glucose levels were determined in whole blood (HemoCue AB, Ängelholm,

Sweden) obtained by cutting the tip of the tail ($t=0$). Then, insulin (Actrapid; Novo Nordisk, Gentofte, Denmark) in a dosage of 1 U per kg body weight diluted in 0.9% sterile NaCl containing bovine serum albumin (g/ml) (fatty acid free; Boehringer-Mannheim) was injected i.p. Exactly 30 min later ($t=30$), blood glucose levels were determined again. The glycemic response to the weight-adjusted dose of insulin, expressed as the percentage fall in glucose ($[\text{glucose}]_{t=0} - [\text{glucose}]_{t=30} / [\text{glucose}]_{t=0} \times 100\%$) was used as an insulin sensitivity marker. After the insulin sensitivity test 100 mg glucose was injected i.p. in order to reduce hypoglycemia.

2.4. Lipids and lipoproteins

At the end of the study, non-fasting plasma levels of total cholesterol (TC) and triglycerides (TG) were measured enzymatically on a Cobas Fara analyzer using reagents from Roche Diagnostica (Copenhagen, Denmark). Lipoprotein cholesterol distributions were evaluated in individual samples from five mice in each group after fractionation by size exclusion chromatography as previously described [7].

2.5. Quantification of atherosclerosis

At 6 months of age (4-month study period) the mice were anesthetized with pentobarbital (5 mg/ml) and exsanguinated by withdrawing the maximum amount of blood from the right ventricle. The blood was centrifuged, and plasma was stored at -20 °C for later lipid measurements. The mice were flushed with isotonic saline and then perfusion-fixed (phosphate-buffered 4% formaldehyde, pH 7.2) at ~100 mmHg via the left ventricle and immersed in the fixative for 6 h. The heart, including the aortic root, was removed and cut transversely [8], and embedded in paraffin. The aortic root was cross-sectioned serially at 4- μ m intervals. Five sections taken at 80- μ m intervals, spanning 320 μ m of the aortic root from the commissures of the aortic leaflets and outward, were stained with orcein (for elastic tissue) and evaluated microscopically. Plaque area, delimited by the lumen and the internal elastic membrane, was measured blindly by the same person (LGL) using computer-assisted image analysis (Sigma Scan Pro, San Rafael, CA, USA), and the mean of the five measurements was determined (mean plaque area).

2.6. Obesity

Four sets of fat pads (inguinal, periovarial, renal and retroperitoneal fat pads) were dissected and weighed. The renal and the retroperitoneal fat pads were taken out in one piece. An adiposity index (AI) was computed for each mouse ($\Sigma(\text{fat pads})/\text{body weight} \times 100\%$).

2.7. Statistical analysis

Values are reported as mean±S.E.M. The Kruskal–Wallis test (non-parametric test) was used to determine differences between means of the three groups of mice. A non-parametric correlation test, Spearman's rho (r_s), was used to determine correlations between the different parameters. The Mann–Whitney *U*-test or the independent *t*-test was used to compare the groups pair-wise. $P < 0.05$ was considered statistically significant. The statistical analysis was done using the SPSS-software version 10.0 for Windows (Chicago, IL, USA).

3. Results

Of the 68 mice that entered the study, 14 mice (three controls (15%), five food-restricted (25%) and six GTG-injected (21%)) died during or immediately after blood sampling (5-h fasting values). Body weight did not differ between those mice who died and those who survived (control $P=0.11$, food-restricted $P=0.65$, and GTG-injected $P=0.15$).

3.1. Body weight, food consumption and obesity

The mean body weight in the three groups did not differ at baseline ($P=0.31$) but did at the end of the study ($P < 0.0001$, Fig. 1). The GTG-injected mice were divided into two groups, responders and non-responders, according to their weight gain. Responders ($n=12$) were characterized by an extraordinary long-term weight gain resulting in a final body weight and AI markedly larger than those of control mice (bodyweight and AI > mean of controls + 5S.D.) (Figs. 1, 2A,B). The non-responding GTG-injected

mice remained lean ($n=10$) and followed a growth curve similar to that of control mice (Fig. 1). Nearly all GTG-injected mice experienced a weight loss during the first week after the injection. However, the mean weight loss was larger in the responding mice than in the non-responders (4.0 ± 0.7 vs. 1.8 ± 1.9 g, $P=0.0025$).

A marked reduction in food consumption was seen in the first week after GTG injection (Fig. 3). Thereafter, food intake increased rapidly to a level similar to control mice. Throughout the rest of the study the consumption curves of the control and GTG-injected mice did not differ ($P=0.87$). Measurable hyperphagia was not observed in cages containing GTG-injected mice. It should, however, be noted that non-responding and responding mice were housed together.

3.2. Glucose, insulin, and glycated hemoglobin

GTG-injected mice had significantly higher 5-h fasting glucose and insulin values than control and food-restricted mice ($P < 0.0001$, Table 1). Body weight and AI correlated positively and significantly with fasting insulin ($n=22$, $r_s=0.60$ (weight), $r_s=0.55$ (AI), $P < 0.001$) and fasting glucose ($n=22$, $r_s=0.53$ (weight), $r_s=0.56$ (AI), $P < 0.001$).

Between control and food-restricted mice, fasting glucose ($P=0.24$) and insulin ($P=0.48$) did not differ. Glycated hemoglobin was similar in all groups (data not shown).

3.3. Insulin sensitivity test

The glycemic response to i.p. injection of a weight-adjusted dose of insulin differed significantly between groups ($P < 0.0001$, Table 1). A less and more pronounced

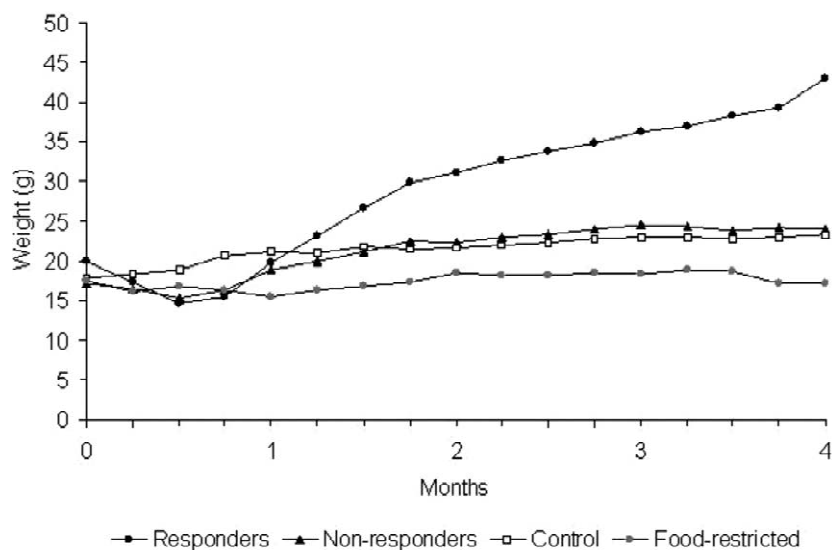


Fig. 1. Body weight in apoE^{-/-} mice throughout the 4 months study period. The final body weight of the responding GTG-injected, non-responding GTG-injected, control and food restricted mice were 40.6 ± 6.9 , 24 ± 2.1 , 23.4 ± 0.24 and 17.1 ± 0.31 g, respectively ($P < 0.001$).

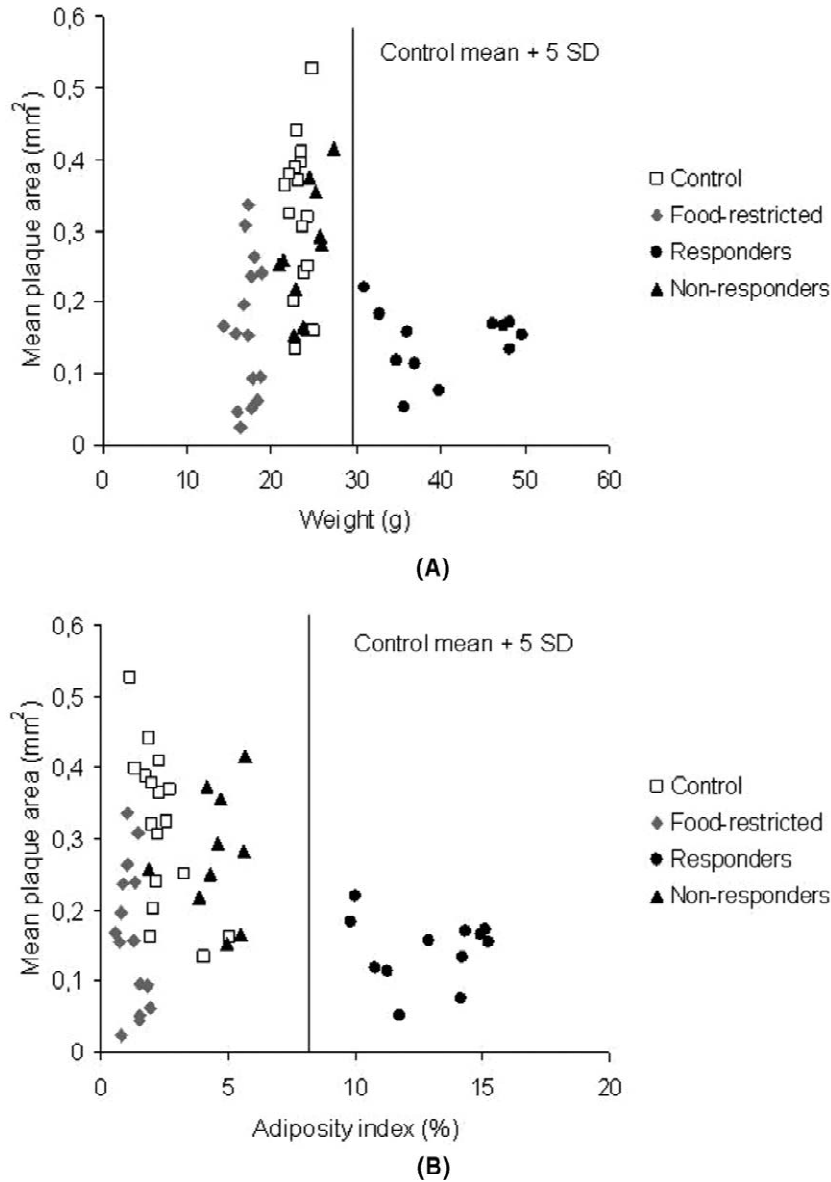


Fig. 2. (A) Atherosclerotic plaque area as a function of body weight. (B) Atherosclerotic plaque area as a function of adiposity index (AI). The AI of the responding GTG-injected, non-responding GTG-injected, control and food-restricted mice were 12.9, 4.5, 2.4 and 1.2%, respectively ($P < 0.0001$). Those GTG injected mice that gained weight more than control mean ± 5 S.D. were exactly the same as those with an AI larger than control mean ± 5 S.D.

glucose lowering response was seen in GTG-injected mice (indicating insulin resistance) and food-restricted mice (indicating insulin hypersensitivity), respectively, compared to control mice (Table 1). No mice died during the procedure.

3.4. Plasma lipids and lipoproteins

The plasma TC levels in the food-restricted, control and GTG group were 16.9 ± 0.57 , 17.3 ± 0.88 and 19.8 ± 0.88 mmol/l, respectively, and these concentrations differed significantly ($P = 0.013$). For the obese mice the plasma TC correlated positively and significantly with body weight ($n = 22$; $r_s = 0.55$, $P = 0.034$). The plasma TG levels in the

food-restricted, control and GTG group were 0.8 ± 0.2 , 0.58 ± 0.16 and 1.1 ± 0.39 mmol/l, respectively ($P = 0.005$).

Lipoprotein-cholesterol distributions were determined by size exclusion chromatography (Fig. 4). The VLDL- and LDL-cholesterol peaks were higher in GTG-injected mice and lower in food-restricted mice compared with control mice. There was, however, no significant difference between the lipoprotein distributions of the three groups. The HDL-cholesterol was barely detectable in any of the groups.

3.5. Atherosclerosis

Atherosclerosis was evaluated in the aortic root. Ad-

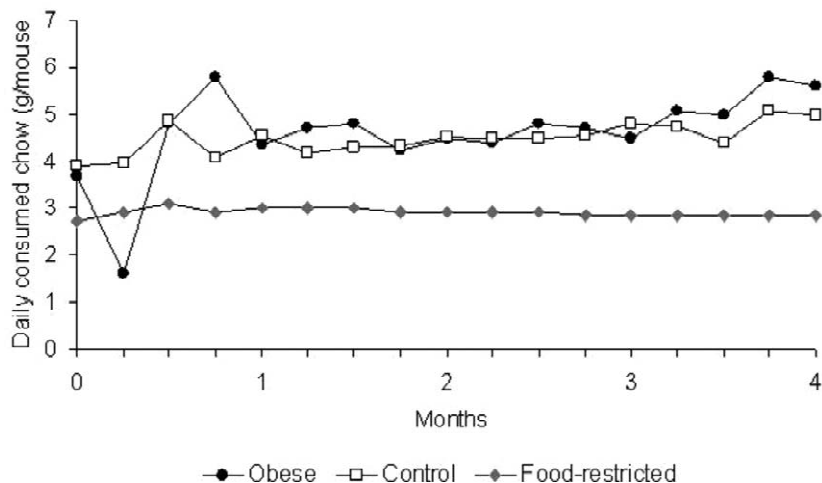


Fig. 3. Mean food-consumption per mouse per day. The obese and the control group were fed ad libitum, whereas the lean mice were strictly kept at a daily amount of food (25% food reduction compared to the control).

vanced atherosclerotic plaques were observed in all groups. No plaque rupture or luminal thromboses were seen.

Mean plaque area differed significantly between the three groups of mice ($P=0.005$, Fig. 5), despite a substantial overlap in plaque area among the groups. Compared to control mice, GTG-injected mice ($P=0.004$) and food-restricted mice ($P<0.0001$) had smaller plaques. The smaller plaque area in the GTG-injected mice was only seen in responders (Fig. 5). There was no relationship between the initial weight loss and plaque size in responders, nor in non-responders (data not shown). The mean plaque area was similar in GTG-injected and food-restricted mice ($P=0.25$, Fig. 5).

In GTG-injected mice, mean plaque area correlated

negatively with body weight ($n=22$, $r_s=-0.55$, $P=0.013$; Fig. 2A), obesity (AI) ($n=22$, $r_s=-0.67$, $P=0.003$; Fig. 2B) and fasting insulin level ($n=22$, $r_s=-0.38$, $P=0.022$).

4. Discussion

This study shows for the first time that GTG injection in atherosclerosis-prone apoE^{-/-} mice induces obesity, insulin resistance, hyperinsulinemia, hyperglycemia and hypertriglyceridemia, i.e., type 2 diabetes mellitus. Unexpectedly, these obese and diabetic mice developed significantly less atherosclerosis than the lean non-diabetic control mice. The food-restricted mice also developed less atherosclerosis.

The neurotoxic glucose analogue GTG has been used for decades to induce obesity in mice [6]. Just a single peripheral GTG injection destroys within 24 h leptin receptor-positive hypothalamic neurons, including those regulating food intake (satiety) and energy expenditure (metabolic rate and heat production), resulting in subsequent development of obesity, leptin resistance (hyperleptinemia) and insulin resistance (hyperinsulinemia)-features that also characterize human obesity [9–12]. An acute weight loss is usually seen during the first week after GTG injection but most of the responding mice will be obese, hyperleptinemic and hyperinsulinemic just 1 month later [9,10]. We observed an increase in body weight in our responding apoE^{-/-} mice. They were severely obese, hyperinsulinemic and hyperglycemic 4 months after the GTG injection. Similarly, Hirano et al. recently reported that plasma insulin, glucose and cholesterol levels were elevated by 213, 23, and 51%, respectively, in obese apoE^{-/-} mice 14 weeks after GTG injection [13].

In healthy apoE^{-/-} mice, no major fluctuations are seen in plasma glucose, insulin, cholesterol and triglyceride

Table 1

Fasting plasma values of insulin and glucose and the results of insulin sensitivity test (IST): values of insulin and glucose were obtained 3 weeks before the end of the study on 5-h fasting blood samples; IST was performed 1 week before the end of the study in non-anesthetized mice fasted for 18 h

	Insulin* (ng/ml)	Glucose* (mmol/l)	IST* (%)
Food-restricted ($n=15$)	0.39±0.05	7.35±0.26	0.71±0.03
Control ($n=17$)	0.43±0.04	7.80±0.26	0.61±0.03
GTG ($n=22$)	1.85±0.34	10.1±0.37	0.47±0.02
Non-responders ($n=10$)	1.12±1.37	9.03±1.44	0.51±0.10
Responders ($n=12$)	2.45±1.58	11.03±1.4	0.44±0.10

Data are presented as mean±S.E.M.

* $P<0.001$ (comparison of the mean differences between the three groups; GTG (responders+non-responders), control and food-restricted).

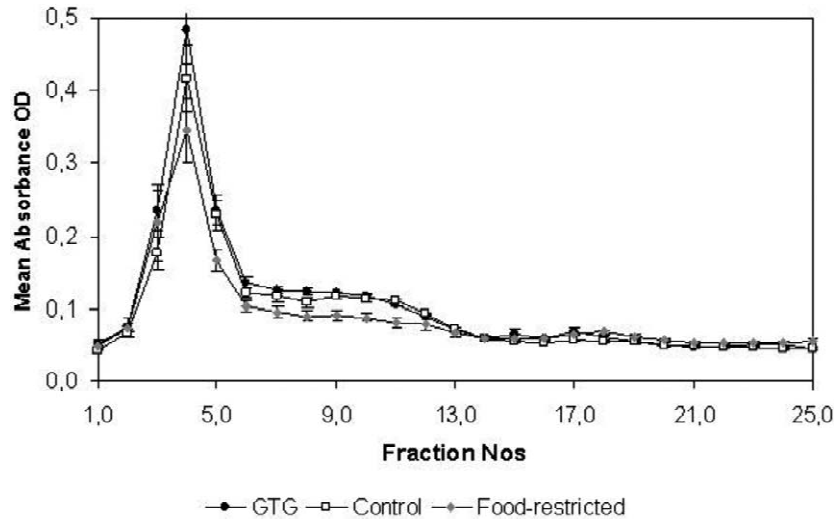


Fig. 4. Distribution of plasma lipoprotein cholesterol determined by size exclusion chromatography. Points represent the mean of five chromatographic separations that were performed on plasma from five mice in each group, and the bars represent S.E.M.

levels between 2 and 6 months of age [5,14]. However, no such longitudinal data are available for GTG-treated apoE^{-/-} mice. With the contrasting result in mind (less atherosclerosis despite a seemingly proatherogenic metabolic profile), it would be interesting to know the temporal changes in the glucose and lipid metabolism after GTG-injection.

Most of the non-responders experienced a weight loss immediately after GTG injection. However, the average weight loss of the non-responding GTG-injected mice was less than that observed by the responding mice (1.7 vs. 4.0

g). It seems unlikely that such a short-term weight loss in adult mice protects against subsequent long-term development of atherosclerosis. The lack of correlation between the initial weight loss and plaque size in responders and in non-responders argues against this possibility.

Non-responding GTG-injected mice and control mice developed similar amount of atherosclerosis and significantly more than responders. A systemic obesity-unrelated anti-atherogenic effect of GTG such as regulation of blood pressure therefore seems unlikely. However, the blood GTG levels might have been higher in responders than in

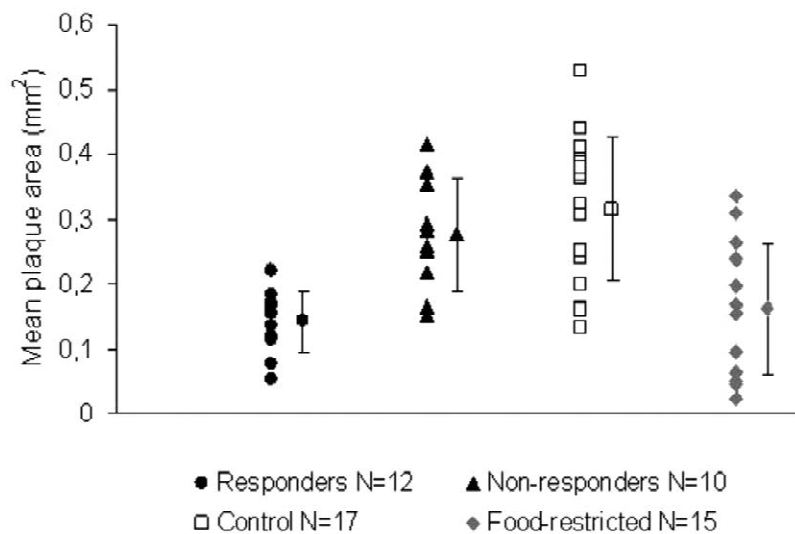


Fig. 5. Atherosclerosis measured in the aortic root (mm²). Mean plaque area (mm²) was 0.14 ± 0.05 , 0.28 ± 0.09 , 0.32 ± 0.11 and 0.19 ± 0.12 in the responding GTG-injected, non-responding GTG-injected, control and food restricted mice, respectively. Mean plaque area \pm 1 S.D. is shown for each group ($P < 0.005$).

non-responders, reflecting the extent of the immediate weight loss and suggesting that GTG is highly toxic and affects the mice in general. However, other effects of GTG such as regulation of blood pressure and other systemic effects have not been described previously. The destruction of hypothalamic neurons is the only known action of GTG [6,11,15].

GTG-induced obesity is usually caused by an increase in energy intake and a decrease in energy expenditure. However, the latter is enough to induce obesity. Pair-feeding experiments of GTG-injected mice have shown that obesity develops without hyperphagia [10,12]. This together with the collective housing of responders and non-responders may explain that hyperphagia was not documented in the GTG-injected mice.

The apoE^{-/-} mouse is a well-established animal model in atherosclerosis research. On standard chow, apoE^{-/-} mice develop severe hypercholesterolemia and advanced atherosclerotic lesions spontaneously throughout the arterial system [5,16]. A high-fat Western-type diet may double the plasma cholesterol level and accelerate the development of atherosclerosis. However, minor changes in total cholesterol and triglyceride levels, as those seen in the present study, have no documented (anti)-atherogenic impact in this model [17].

The apoE^{-/-} mice, used in the present study, were backcrossed 10 generations into the C57BL/6 background, which is the most used mouse strain in studies determining the relation between obesity and insulin resistance [18–20]. In C57BL/6 mice, diets high in fat [18–20] and/or simple carbohydrates [17] easily induce obesity and insulin resistance. However, using such a dietary approach, we were not able to induce either obesity or insulin resistance in our apoE^{-/-} mice, despite their C57BL/6 genetic background (unpublished data). Nevertheless, the present study documents that the apoE^{-/-} mouse is not inherently resistant to diet-induced obesity and insulin resistance, because both conditions are prone to develop, even in chow-fed mice, after GTG injection. The relevance of this observation is strengthened by the positive and highly significant correlation between obesity and markers of insulin resistance (fasting hyperinsulinemia and hyperglycemia) in the GTG-injected mice.

The paradoxical finding of reduced atherosclerosis in obese apoE^{-/-} mice with type 2 diabetes is, however, in agreement with corresponding results obtained in other mouse models. Several genetic models of obesity-linked insulin resistance and type 2 diabetes exist in mice, among others obese (*ob*), diabetic (*db*), fat (*fat*), tubby (*tub*), lethal yellow (*A^y*), and KKA^y mice. Atherosclerosis does not develop spontaneously in any of these mice, and fatty streak formation in the aortic root is not accelerated, compared to their wild-type counterpart, by an atherogenic diet high in fat, cholesterol, and cholate [21]. Most of these genetically obese and insulin-resistant mice strains develop in fact significantly less atherosclerosis on the atherogenic

diet than their lean wild-type controls. Also the LDL receptor-deficient mouse appears to develop less atherosclerosis with diet-induced insulin-resistance, documented by less atherosclerosis in obese, insulin-resistant mice fed high-fat diet than in lean, non-insulin-resistant, fructose-fed mice [22]. Thus, obesity and insulin resistance acquired or of genetic origin, appears to confer relative protection against the development of atherosclerosis in mice.

The reason for this paradoxical protection against atherosclerosis in insulin-resistant mice (versus some strains of rats [23]) is unknown, but an unusual lipoprotein pattern might play a critical role [24]. Insulin resistance and type 2 diabetes are associated with an extremely atherogenic dyslipoproteinemia in humans (high VLDL, low HDL and small dense LDL-particles) but the opposite might theoretically occur with insulin resistance and type 2 diabetes in mice caused by their characteristic non-human-like lipoprotein metabolism. Triglyceride-rich VLDL particles may be so large that they are unable to penetrate into the arterial wall resulting in reduced atherosclerosis, whereas smaller cholesterol-rich particles (IDL/LDL) are much more atherogenic. This probably explains the atheroprotective effect of alloxan-induced type 1 diabetes observed in cholesterol fed rabbits [25]. In the present study, however, there was no change in lipoprotein-cholesterol distribution that could explain the lesser amount of atherosclerosis observed in our obese mice with type 2 diabetes.

Compared with ad libitum feeding, 40% food restriction increases longevity substantially in mice [26]. In rabbits food restriction does not lead to any regression of atherosclerosis [27]. Only a few studies have addressed the effect of food restriction on the development of atherosclerosis in mice despite the fact that many drug regimens claimed to be atheroprotective in mice also inhibit normal growth [28–30].

In the present study, 25% food restriction alone reduced the development of atherosclerosis significantly in apoE^{-/-} mice compared with those fed chow ad libitum. This anti-atherogenic effect of food restriction could not be explained by beneficial changes in plasma total cholesterol and triglyceride levels. The same was observed in a newly published study [31], which also raises the hypothesis that the atheroprotective effect of food restriction is linked to reduced oxidative stress in the arterial wall [31]. Although the anti-atherogenic effect of restricted feeding is not necessarily seen with reduced caloric intake caused by a drug, it should be considered as a possibility when an anti-atherogenic intervention also retards or prevents the normal growth of a mouse.

A limitation of the study is that we used only female mice. The advantage of using female mice is that they develop larger atherosclerotic plaques in the aortic root than male mice [32]. However, the opposite might be true when the total arterial tree is examined [33]. GTG is

shown to have equal effect in male as well as in female mice [9,34] so in that matter we have no reason to believe that our results would have been different using male mice.

5. Conclusions

GTG-injected ad libitum fed apoE^{-/-} mice developed type 2 diabetes, including obesity, insulin resistance, hyperinsulinemia, hyperglycemia and hypertriglyceridemia. Contrary to our expectations, these obese and diabetic mice developed less atherosclerosis than lean non-diabetic control mice. Food-restricted mice also developed less atherosclerosis. These findings may question the usefulness of mouse models in studying the relation of obesity and/or type 2 diabetes to atherosclerosis, and the relevance of results obtained in apoE^{-/-} mice with reduced weight gain during intervention.

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