

Rosiglitazone Reduces the Accelerated Neointima Formation After Arterial Injury in a Mouse Injury Model of Type 2 Diabetes

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Background—Hyperglycemia (HG) and hyperinsulinemia (HI) may be factors enhancing the atherosclerotic complications of diabetes. We hypothesized that specific feeding of C57BL/6 apolipoprotein (apo) E^{-/-} mice would alter their metabolic profiles and result in different degrees of neointima (NI) formation. We additionally hypothesized that an insulin-sensitizing agent (rosiglitazone) would prevent the development of type 2 diabetes and reduce neointima formation after carotid wire injury measured at 28 days.

Methods and Results—Fasting glucose and insulin levels were elevated in the Western diet (WD) group, with a trend toward higher insulin levels and euglycemia in the fructose diet (FD)-fed mice. NI formation was exaggerated in the WD group compared with the FD or chow control groups. In the WD mice given rosiglitazone, glucose and insulin levels remained normal and NI formation was significantly reduced, as was NI macrophage content.

Conclusions—These findings demonstrate that apoE^{-/-} mice fed a WD develop type 2 diabetes with an exaggerated NI response to injury. FD mice maintain euglycemia but develop insulin resistance, with an intermediate degree of NI growth compared with chow diet controls. Rosiglitazone prevents the development of hyperglycemia and hyperinsulinemia and normalizes the insulin release profile in the apoE^{-/-}, WD-fed mouse and significantly reduces NI formation by 65% after carotid wire injury while reducing macrophage infiltration. These data support the hypothesis that type 2 diabetes in the setting of elevated cholesterol accelerates the response to vascular injury and suggest that agents that improve insulin sensitivity may have therapeutic value in reducing restenosis in type 2 diabetes. (*Circulation*. 2003;108:1994-1999.)

Key Words: angioplasty ■ drugs ■ hypercholesterolemia ■ diet ■ diabetes mellitus

Atherosclerotic vascular disease is a major cause of increased morbidity and mortality in humans with type 2 diabetes mellitus.^{1,2} The present trend of increased obesity is predicted to significantly increase the incidence of type 2 diabetes in the United States population.³ Although the prevalence of atherosclerosis is increased in type 2 diabetes, the underlying mechanisms responsible remain poorly understood.⁴ The in vivo study of the interactions and contributions of hyperglycemia, hyperinsulinemia, and hypercholesterolemia in the development of atherosclerosis and the response to vascular injury in type 2 diabetes has been limited by available animal models that develop all of these metabolic abnormalities.

The C57BL/6 mouse strain has been shown to develop diet-induced type 2 diabetes and atherosclerosis when fed a high-fat, Western diet (WD) for prolonged periods of time.^{5,6}

These mice do not develop significant hypercholesterolemia, and the spontaneous lesions that develop are immature and have a restricted anatomic distribution.^{5,7} The generation of the apolipoprotein E-deficient (apoE^{-/-}) mouse on the C57BL/6 background has provided a model that develops severe hypercholesterolemia and atherosclerosis throughout the arterial tree that is accelerated on a WD.⁸⁻¹⁰ In addition, a high-fructose diet (FD) has been reported to induce hyperinsulinemia, with insulin resistance and euglycemia in rats.^{11,12} Rats, however, generally do not develop hypercholesterolemia and do not develop significant atherosclerosis.^{13,14} The normal pattern of insulin secretion has been shown to be biphasic both in isolated perfused preparations of rat pancreatic islets in vitro and in humans.¹⁵⁻¹⁷

Peroxisome proliferator-activated receptor-γ has been shown to be expressed in many of the cells that play a role in

Received September 24, 2002; de novo received March 20, 2003; revision received June 16, 2003; accepted June 17, 2003.

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This work was supported by an unrestricted grant from GlaxoSmith-Kline.

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Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000092886.52404.50

the response to vascular injury and modulates the actions that are thought to initiate neointimal (NI) growth, including inflammation.^{18–24} Three different thiazolidinediones, rosiglitazone, pioglitazone, and troglitazone, have been shown to prevent spontaneous atherosclerosis in the aorta of LDLR^{-/-} mice or in balloon-injured rat carotid arteries but have not been studied in a model of arterial injury in the setting of hypercholesterolemia.^{25–27} This is important, because these agents, which are ligands for peroxisome proliferator-activated receptor- γ , are used in the treatment of patients with type 2 diabetes who often have concomitant hypercholesterolemia and symptomatic, obstructive coronary atherosclerosis.

Based on these data, we hypothesized that C57BL/6 apoE^{-/-} mice fed a WD would develop hypercholesterolemia with a metabolic profile of hyperinsulinemia and hyperglycemia with an insulin release profile consistent with type 2 diabetes whereas apoE^{-/-} mice fed a FD would develop hypercholesterolemia with hyperinsulinemia but euglycemia and an insulin release profile consistent with the metabolic syndrome. We additionally hypothesized that in the setting of carotid wire injury, NI growth would be accelerated in the WD mice and that treatment with an insulin-sensitizing agent (rosiglitazone) would prevent the development of type 2 diabetes and reduce NI formation after carotid wire injury at 28 days in the apoE^{-/-}, WD-fed mouse.

Methods

Animals

Female C57/BL6 apoE^{-/-} mice 8 to 10 weeks of age (18 to 20 g; The Jackson Laboratory, Bar Harbor, Me) were used for these experiments. Animals were handled in compliance with the *Guiding Principles in the Care and Use of Animals*. Protocol approval was obtained from the Animal Research Committee of the University of Virginia Health System.

Mouse Injury Model

The mouse carotid artery wire injury model of Lindner et al²⁸ was used with minor modification, as we have previously published.^{29,30} Mice (N=10 per group) were fed either a WD (TD 88137, Harlan-Teklad; containing 21% fat by weight, 0.15% by weight cholesterol, and 19.5% by weight casein without sodium cholate), a FD (TD 96130, Harlan-Teklad; containing 13% of calories from fat, 67% from carbohydrates, 20% from protein), a chow diet (CD), or a WD with rosiglitazone (10 mg/kg per day, GlaxoSmithKline) for 1 week before and 4 weeks after carotid injury.

Quantitative Histopathology

The arterial segments were dehydrated in ethanol and xylene and embedded in paraffin. Sections (5 μ m thick) were stained by the Movat method.³¹ Histomorphometric analysis was performed by individuals blinded to type of diet. For quantitative histopathologic comparisons, the mean of 10 sections was taken. The area of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined by planimetry using Image Pro Plus 3.0 (Media Cybernetics), and the lumen area, plaque area, medial area, intima to media ratio, and overall vessel area were calculated. NI area was calculated by subtracting lumen area from the IEL area, and medial area was determined by subtracting the IEL area from the EEL area. Arterial size was measured by tracing the circumference of the EEL.

Immunocytochemistry

Sections were stained for macrophage/foam cells using an anti-mouse macrophage mAb F4/80 (Accurate Chemical and Scientific Corp) or for smooth muscle actin-positive cells using mAb 1A4 (Dako Corp). For quantitative immunocytochemical comparisons of macrophage content or smooth muscle cell content, sections were digitized and the number of positively stained pixels were counted and normalized to total NI and medial area using Image Pro Plus 3.0 (Media Cybernetics).

Blood Chemistry

Blood glucose levels were assessed before initiation of diets, after 1 week of diet, and at the time of euthanasia by glucometer (Accu-check Advantage; Roche). In addition, fasting glucose, insulin, and lipid panels were assessed at the time of euthanasia after 5 weeks of the respective diets. Blood samples at the time of euthanasia were drawn by cardiac puncture into serum separator tubes (Becton-Dickinson). Lipid levels were determined by the University of Virginia Clinical Pathology Laboratory.

Pancreatic Islet Isolation

At the time of euthanasia, before perfusion fixation, the pancreas of each mouse was removed and prepared for histology or islet cells isolated for glucose perfusion and insulin release kinetics. Mouse pancreatic islets were isolated using a method modified from previously published protocols.^{32,33} Briefly, after exposition of the pancreas, the common duct of bile was cannulated and injected with Hanks' solution containing 0.7 to 1 mg/mL Collagenase P (Roche Molecular Biochemicals) and the dissected pancreas was digested at 37°C. Pancreatic islets were separated from pancreatic digest by Ficoll density gradients (Sigma). Islets were individually picked, washed, and cultured overnight at 37°C in 5% CO₂ in M199 medium (Life Technologies) supplemented with 10% FCS and antibiotics.

Measurement of Insulin Release in a Perfusion System

The isolated pancreatic islets of Langerhans from the mice were subjected to overnight culture in RPMI1640 medium (GIBCO) supplemented with 10% FBS in a tissue culture incubator (37°C, 5% CO₂). After overnight culture, 100 islets from each diet-fed group of mice were transferred to a perfusion chamber. The temperature was maintained at constant 37°C. The islets were perfused at a rate of 1 mL/min using a multichannel peristaltic pump (Harvard Instruments) with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4), continuously gassed with 95% oxygen and 5% carbon dioxide, and supplemented with 20 mmol/L HEPES, 0.1% BSA, and glucose as required. The preliminary perfusion was performed for 30 minutes with KRB containing 3.0 mmol/L glucose to obtain stable baseline insulin secretion. The perfusion medium was then rapidly replaced by KRB containing 30 mmol/L glucose and sustained for 60 minutes. The perfusion medium was switched back to KRB containing 3.0 mmol/L glucose for another 30 minutes. The perfusate from each chamber was collected at 1-minute intervals, and 25 μ L of perfusate from each collected sample was analyzed for insulin concentration (microgram per milliliter) using EIA (ALPCO) with crystalline mouse insulin as standard. The insulin secretion profiles from islets of each of the 3 diet-fed groups were generated by plotting the perfusate insulin contents against the duration of perfusion.

Statistical Analysis

Statistical analysis was performed using NCSS 97. Data are reported as the number of carotid arteries in each group, and plaque area and intima to media ratio are expressed as mean \pm SD. Data were compared using ANOVA and Student's *t* test to evaluate 2-tailed levels of significance.

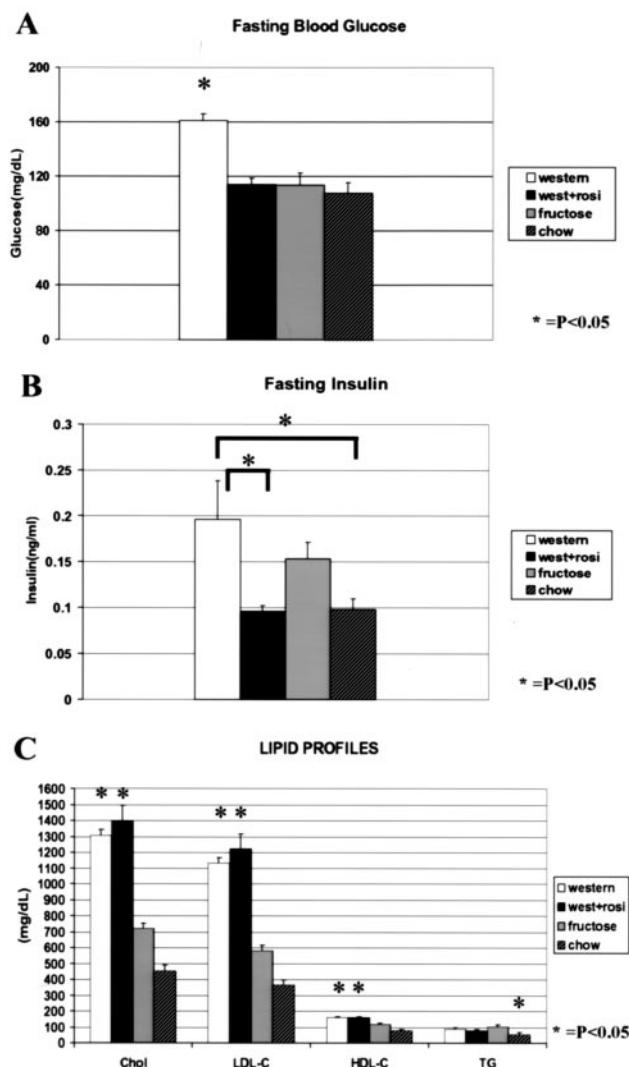


Figure 1. Metabolic profiles. A, Fasting blood glucose in the WD mice vs FD or CD after 5 weeks of diet. Note the significantly higher fasting glucose levels in the WD group vs FD or CD (*P<0.05). Rosiglitazone therapy in the WD-fed mice results in maintenance of normoglycemia. B, Fasting insulin levels were significantly higher in the WD vs CD group, with a nonsignificant trend toward higher insulin levels in the FD group after 5 weeks on feed. Rosiglitazone therapy in the WD mice results in insulin levels similar to those of the CD group. C, Total cholesterol, LDL, and HDL levels in the WD, FD, and CD groups. A graded elevation in total cholesterol, LDL, and HDL levels was observed in the WD vs FD and CD groups. Triglyceride levels were elevated in the WD and FD animals, and WD mice with rosiglitazone had levels that were no different than the WD-only group.

Results

Metabolic Profiles and Insulin Release Kinetics

Baseline glucose levels were normal in all groups before initiation of diets and after 1 week of feeding at the time of carotid wire injury (data not shown). After 5 weeks on diet, at the time of euthanasia (Figure 1A), fasting glucose levels were higher in the WD group versus FD or CD groups whereas glucose levels in mice fed a WD plus rosiglitazone remained normal. Fasting insulin levels were significantly higher in the WD group compared with CD group, with a

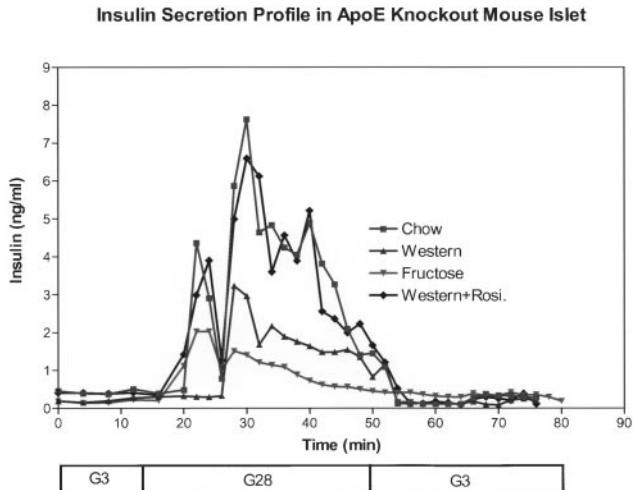


Figure 2. Pancreatic islet insulin release kinetics. Insulin release profiles from pancreatic islet cells after perfusion of glucose demonstrating loss of the first peak and an attenuated second peak in WD animals, consistent with a pattern of type 2 diabetes with normalization of this pattern in the WD animals treated with rosiglitazone. Note the attenuation of both first and second peaks in FD animals, consistent with insulin resistance and a normal biphasic release profile in CD animals (n=10 animals per group with 100 islets isolated per diet-fed group for perfusate insulin levels).

nonsignificant trend toward higher insulin levels in the FD group at 5 weeks (Figure 1B). Insulin levels in the WD group with rosiglitazone remained normal (Figure 1B). Insulin release profiles from pancreatic islet cells with perfusion of glucose demonstrated loss of the first-release peak and an attenuated second-release peak in WD animals, consistent with a pattern of type 2 diabetes. In contrast, rosiglitazone-treated, WD-fed mice had normal release kinetics, similar to the CD animals, which demonstrated a normal biphasic release profile. Attenuation of both first and second peaks was observed in FD animals, consistent with a pattern of insulin resistance (Figure 2).

Lipid Profiles

A graded elevation in total cholesterol, LDL, and HDL levels was observed in the WD versus FD and CD groups. Triglyceride levels were elevated in both the Western and FD animals (Figure 1C). Total cholesterol, LDL, and HDL levels were elevated in the WD with rosiglitazone group to a level that was equal to that seen in the WD-alone group (Figure 1C).

Histomorphometry

There were no differences in the extent of injury between any of the groups as defined by number of elastic laminae broken (data not shown). At 28 days after carotid wire injury, NI formation was significantly greater in the WD group compared with the FD group ($31\ 000 \pm 7000 \mu\text{m}^2$ versus $11\ 000 \pm 2500 \mu\text{m}^2$, $P \leq 0.05$, n=10 per group). The FD group had significantly greater NI than the CD group ($11\ 000 \pm 2500 \mu\text{m}^2$ versus $5130 \pm 1000 \mu\text{m}^2$, $P \leq 0.05$, n=10 per group) (Figure 3A). There was a significant 65% reduction in NI formation in the WD group treated with rosiglitazone com-

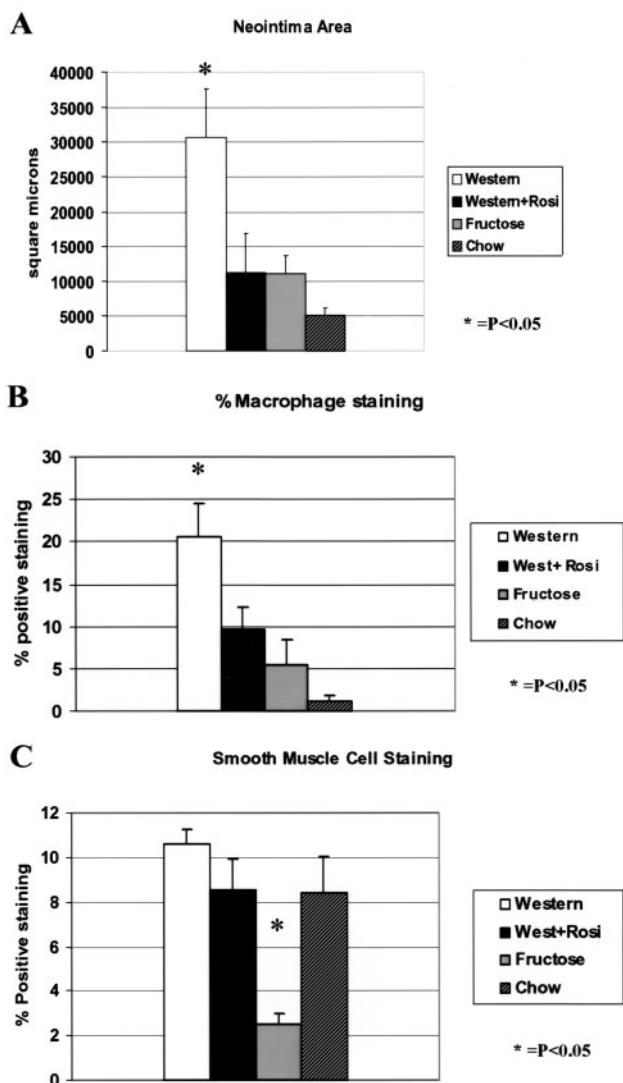


Figure 3. Histomorphometry and immunohistochemistry. A, Quantitative histomorphometry of plaque area in injured carotid arteries 4 weeks after wire denudation and 5 weeks on a WD, FD, CD, or WD with rosiglitazone. Note the markedly increased neointimal growth in the apoE^{-/-} mice fed a WD compared with the FD or CD mice, *P<0.05. Also, note the significantly more robust NI growth in the FD group vs the CD group, *P<0.05. B, Quantitative immunocytochemistry of macrophage infiltration into the wall of injured carotid arteries 4 weeks after denudation and 5 weeks on respective diets. Note the marked reduction in percent area occupied by macrophages in the WD with rosiglitazone group compared with the WD group, *P<0.05. In addition, the FD and CD groups have significantly less macrophage staining compared with the WD group. C, Smooth muscle cell staining demonstrating significantly less staining in the FD group compared with the other groups, *P<0.05.

pared with the WD group ($11\,000 \pm 5000 \mu\text{m}^2$ versus $31\,000 \pm 6000 \mu\text{m}^2$, $P \leq 0.05$, $n=10$ per group). Macrophage content in the injured vessel wall was significantly reduced by 52% in the WD group treated with rosiglitazone compared with WD alone ($9.5 \pm 2\%$ versus $20 \pm 4\%$, $P \leq 0.05$, Figure 3B). Fewer macrophages were seen in the FD and chow groups compared with the WD group ($5.5 \pm 2\%$ and $1 \pm 0.8\%$, $P \leq 0.05$, Figure 3B). There was also significantly less staining for smooth muscle cells in the FD group compared with

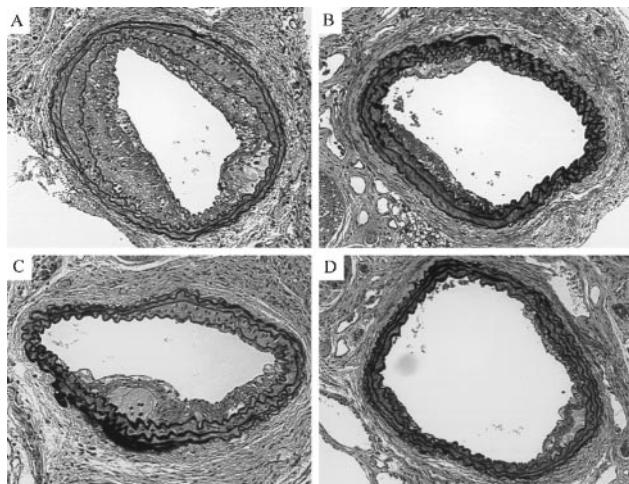


Figure 4. Representative examples of Movat-stained injured left carotid arteries (LCA) from a mouse fed a WD showing robust NI formation (A), a mouse fed a WD with rosiglitazone treatment illustrating significantly less NI growth (B), a mouse fed a FD with moderate NI growth (C), and a mouse fed CD showing minimal NI growth (D). Magnification $\times 200$.

the other groups (Figure 3C). Representative examples of MOVAT-stained arteries from each group are shown in Figures 4A through 4D, immunostaining for macrophages is shown in Figures 5A through 5D, and smooth muscle cells are shown in Figures 6A through 6D. There was no significant difference in either media or EEL areas between groups (data not shown).

Discussion

This is the first study to document a range of metabolic profiles consistent with type 2 diabetes and insulin resistance in C57BL/6 apoE^{-/-} mice fed various diets. We show graded NI formation after arterial injury being most robust in the setting of type 2 diabetes plus hyperlipidemia. On a CD, the apoE^{-/-} mouse develops mild but significant hypercholester-

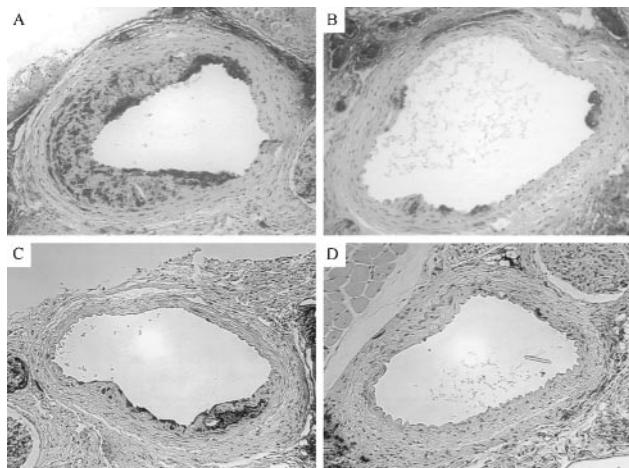


Figure 5. Macrophage content. Representative immunostaining for macrophages using the F4/80 anti-mouse macrophage mAb from a mouse fed a WD (A), a mouse fed a WD with rosiglitazone (B), a mouse fed a FD (C), and a mouse fed CD (D). Magnification $\times 200$.

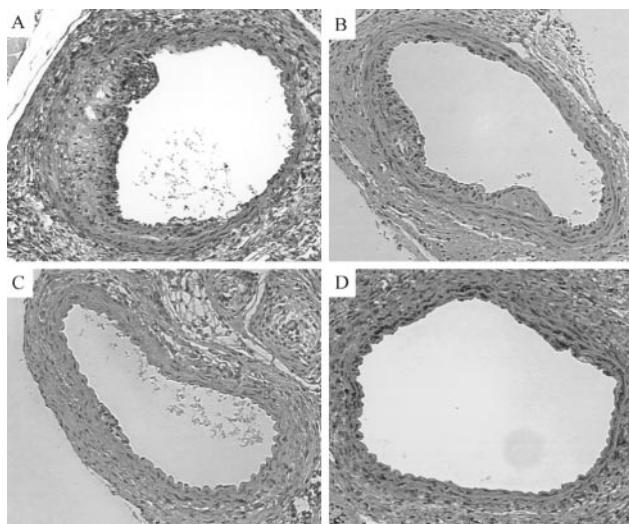


Figure 6. Smooth muscle cell content. Representative immunostaining for smooth muscle cells from a mouse fed WD (A), a mouse fed WD with rosiglitazone (B), a mouse fed FD (C), and a mouse fed CD (D). Magnification $\times 200$.

olemia with elevated LDL cholesterol levels. The fasting glucose and insulin levels on this diet remain in the normal range, and the dynamic insulin release from isolated pancreatic islet cells in the face of varying glucose concentrations also remains normal, characterized by preservation of the first and second insulin release peaks. The response to arterial injury at 28 days is minimal.

The intermediate injury response observed in the FD-fed apoE^{-/-} mice that develop insulin resistance is concordant with a recent report in humans where in-stent restenosis was significantly more common in patients with the metabolic syndrome undergoing percutaneous coronary interventions.³⁴ Arterial injury in the FD group results in a 2-fold increase in NI growth compared with the CD group. In contrast, the WD mice develop more severe hypercholesterolemia, markedly elevated LDL cholesterol levels, and modest increases in triglycerides, as has been previously reported, while developing the most exaggerated injury response.^{9,10}

In the setting of arterial injury, the apoE^{-/-} mouse on a WD develops robust NI formation. This may be in part attributable to the hyperglycemia, hyperinsulinemia, and increased inflammatory response to injury compared with the FD and CD mice. This is supported by the significant 65% reduction in NI formation seen in WD mice treated with rosiglitazone and a 53% reduction in macrophage content. Rosiglitazone therapy of WD-fed mice reduced NI formation and macrophage content that approached that of the FD group but not that of the CD mice. This could be the result of the markedly elevated lipid levels that were not reduced by treating with rosiglitazone. The exaggerated injury response in the WD group is most likely a result of an interaction of these abnormal metabolic factors and inflammation seen on this diet in the apoE^{-/-} mouse.

The development of diabetes on a WD was recently described in the LDL receptor-deficient (LDLR^{-/-}) mouse. However, there was no increase in spontaneous atherosclerosis in the aorta compared with LDLR^{-/-} mice on a FD.³⁵ In

addition, compared with the development of insulin resistance, as determined by insulin release profiles in our apoE^{-/-} mice fed a FD, the LDLR^{-/-} mice did not develop insulin resistance while on a FD.³⁵ Previous studies in the LDLR^{-/-} mice have shown a reduction in lesion formation in male mice treated with thiazolidinediones but not female mice, as we report in our experiments.^{25,26} It is important to note that these studies evaluated spontaneous atherosclerosis in the aortic cusp and aorta in contrast to the model of arterial injury and carotid lesion formation in our experiments. It has recently been shown that injury-induced NI hyperplasia and diet-induced spontaneous atherosclerosis are controlled by distinct sets of genes and responses to each can vary within and between mouse strains.³⁶

In summary, we demonstrate that apoE^{-/-} mice fed a WD develop severe hypercholesterolemia and a metabolic profile consistent with type 2 diabetes and have an exaggerated response to arterial injury. The development of type 2 diabetes in the WD-fed apoE^{-/-} mouse can be prevented by rosiglitazone treatment, and NI formation and macrophage content can be significantly reduced. This model thus provides a valuable tool to study the interaction between atherosclerosis, diabetes, and inflammation.

Acknowledgments

This work was supported by an educational grant from GlaxoSmithKline, NIH/NHLBI Training Grant T32 HL-07355 (Dr Phillips, PI), Grant PO1-55798 (to Dr Nadler), NIH Grant DK-55240 (to Dr Chen), and the Iacocca Foundation (to Dr Yang).

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