Vascular effects of a low-carbohydrate high-protein diet

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The cardiovascular complications of obesity have prompted interest in dietary interventions to reduce weight, including low-carbohydrate diets that are generally high in protein and fat. However, little is known about the long-term effects of these diets on vascular health. We examined the cardiovascular effects of a low-carbohydrate, high-protein diet (LCHP) in the ApoE−/− mouse model of atherosclerosis and in a model of ischemia-induced neovascularization. Mice on a LCHP were compared with mice maintained on either the standard chow diet (SC) or the Western diet (WD) which contains comparable fat and cholesterol to the LCHP. LCHP-fed mice developed more aortic atherosclerosis and had an impaired ability to generate new vessels in response to tissue ischemia. These changes were not explained by alterations in serum cholesterol, inflammatory mediators or infiltrates, or oxidative stress. The LCHP diet substantially reduced the number of bone marrow and peripheral blood endothelial progenitor cells (EPCs), a marker of vascular regenerative capacity. EPCs from mice on a LCHP diet also manifest lower levels of activated (phosphorylated) Akt, a serine-threonine kinase important in EPC mobilization, proliferation, and survival. Taken together, these data demonstrate that in animal models LCHP diets have adverse vascular effects not reflected in serum markers and that nonlipid macronutrients can modulate vascular progenitor cells and pathophysiology.

Vascular disease remains a dominant cause of morbidity and mortality throughout much of the world. The most common form of vascular disease is atherosclerosis, a chronic disorder marked by accumulation of lipid and fibrous material in the vessel wall that can culminate in ischemic tissue injury (1). Atherosclerosis is thought to form as an inflammatory response to a variety of stimuli, including serum lipids that induce endothelial dysfunction and lead to vascular recruitment of leukocytes (2). Similar lesions have been generated in a variety of animal models by increasing dietary fat and cholesterol (3, 4). Recent work has raised the possibility that endothelial progenitor cells (EPCs) may help restore normal vascular function (5, 6). Consistent with this hypothesis, clinical studies suggest EPC number correlates with brachial artery reactivity (7) and inversely with prospectively assessed cardiovascular risk (8). However, the precise role of EPCs in atherogenesis remains poorly defined. In contrast, EPCs are more clearly implicated in enhancing neovascularization in response to tissue ischemia in adults (6, 9), a key component of the healing process after such injury.

The growing epidemic of obesity and concerns over its complications including atherosclerotic vascular disease have prompted interest in interventions such as low-carbohydrate diets. Typically, a reduction in dietary carbohydrate is accompanied by an increase in dietary fat and protein, which proponents suggest could have salutary effects through a net reduction in glycemic load. Indeed, randomized trials suggest low-carbohydrate diets may accelerate weight loss with surprisingly little negative effect on serum markers of cardiac risk such as cholesterol (10, 11). Moreover, a recent study in mice demonstrated that an extremely low-carbohydrate, ketogenic diet induced weight loss disproportionate to the reduction in caloric intake through induction of hepatic FGF21 (12). In contrast, a recent clinical trial has demonstrated that a low-carbohydrate diet impaired flow-mediated vascular reactivity when compared with a low-fat diet, even in the setting of similar weight loss and decreases in blood pressure (13). However, the central clinical question—how such diets affect long-term vascular health—remains largely unaddressed, and we examined this question in murine models.

Results

Effect of LCHP Diets on Atherogenesis. We first investigated dietary effects in ApoE−/− mice, a well-established model of atherosclerosis that recapitulates many features of the human disease (4). We elected to study only male ApoE−/− mice to control for the known effect of gender on atherosclerosis in this strain (14). ApoE−/− mice maintained on a standard chow diet (SC) (65% carbohydrate, 15% fat, 20% protein) develop small amounts of atherosclerosis. In contrast, ApoE−/− mice on the so-called ‘Western’ diet (WD) (43% carbohydrate, 42% fat, 15% protein, and 0.15% cholesterol) develop extensive aortic atherosclerosis including complex plaques similar to those seen in humans (15, 16). To model the maintenance phase of human low-carbohydrate diets where approximately 10–15% of caloric intake is in the form of carbohydrates and there is a compensatory increase in fat and protein content, we used a low-carbohydrate high-protein diet (LCHP) (12% carbohydrate, 43% fat, 45% protein, and 0.15% cholesterol), which contains comparable amounts of fat, cholesterol, and calories as the WD (Table S1). We placed ApoE−/− mice on one of these three diets 1 week after weaning. Milkfat was the primary fat source in all diets. Mice maintained on the LCHP gained less weight than their WD or SC-fed cohorts at the end of the study. After 12 weeks on the diets, percent-weight gain in mice on the LCHP was 28% less than mice on the SC or WD (P < 0.05), which did not differ significantly from each other (Fig. S1). These results are consistent with the greater weight loss observed in clinical trials with low-carbohydrate, high-protein diets.

We examined aortae for the development of atherosclerosis after 6 and 12 weeks on the diets, using an en face analysis of Oil Red-O staining to quantitate atheroma area as a percentage of the aortic luminal area. At 6 weeks, mice on the LCHP had significantly more atheroma than mice on the WD (5.4% vs. 2.2% respectively, P = 0.004; Fig. I4 and B). This difference was maintained after 12 weeks on the diets (15.3% vs. 8.8% respec-


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tively, \( P = 0.013 \). As expected, chow-fed mice had minimal, although quantifiable, amounts of plaque at both 6 (0.5%) and 12 (1.3%) weeks, which was significantly less than that seen in LCHP- and WD-fed mice \( (P \leq 0.01) \) for all pairwise comparisons at 12 weeks. Taken together these data demonstrate the LCHP-fed mice developed more extensive atherosclerosis than WD-fed mice, despite similar dietary fat and cholesterol content, and reduced weight gain.

**Influence of LCHP Diets on Cardiovascular Risk Markers.** To understand the basis for increased atherosclerosis in LCHP-fed mice, we examined sera for recognition of markers of cardiovascular disease (Table 1). As expected, the WD led to an increase in serum total cholesterol \((1,470 \pm 171 \text{ mg/dL})\) compared with SC diet \((359 \pm 28 \text{ mg/dL}, P < 0.001; \text{Table 1})\). Consistent with clinical observations \((10, 11)\), serum total cholesterol was not different on the LCHP \((1,408 \pm 251 \text{ mg/dL})\) compared with the WD, but significantly higher than SC control \((P < 0.001)\). Fractionation of the plasma lipoproteins by fast protein liquid chromatographic (FPLC) revealed no difference in lipoprotein-cholesterol distribution in WD and LCHP-fed mice (Fig. 2C). Similarly, levels of triglycerides, fasting insulin and glucose did not differ between WD- and LCHP-fed mice (Table 1).

Serum levels of oxidized LDL (oxLDL) did not differ significantly between the WD and LCHP-fed mice \((2.47 \pm 1.7 \text{ U/L vs.} 2.14 \pm 1.1 \text{ U/L respectively})\), although both high-fat diets showed increased oxLDL levels compared with SC-fed mice \((0.32 \pm 0.6 \text{ U/L}; \text{Fig. 2B})\). In addition, immunohistochemical labeling with an antibody against 8-oxoguanine, a major product of oxidative stress on DNA, showed a similar level of oxidative damage in the atheromatous lesions of WD- and LCHP-fed mice (Fig. 2C and S2).

Overall, the absence of significant differences in cholesterol, fasting glucose and insulin, or oxLDL levels suggested that neither increased lipids, glucose dysregulation nor oxidative stress were responsible for increased atherosclerosis in the LCHP diet group. Consistent with the decreased intake of carbohydrate, nonfasting glucose was lower in the LCHP group compared with the WD group (Table 1). We did observe an increase in serum levels of nonesterified fatty acids (NEFAs) on the LCHP diet although the role of NEFAs in atherosclerosis remains unclear. However, they are a clinical marker of risk \((17, 18)\) that may increase inflammation \((19)\) that could contribute to atherogenesis.

Inflammation plays an important role in atherogenesis. We therefore examined serum levels of interleukin-6 (IL-6), an indicator of general inflammation, which did not differ among the three cohorts (Fig. 2D). The more specific inflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1), which is induced in atheromatous lesions, implicated in atherogenesis \((20–22)\) and contributes to monocyte recruitment, was significantly increased in the serum of WD-fed compared with SC-fed mice (Fig. 2E). Paradoxically, LCHP-fed mice had MCP-1 levels that were lower than WD-fed mice and did not differ from SC-fed mice. Prior work in mouse models of germline MCP-1 deficiency suggests this reduced MCP-1 should if anything mitigate atherogenesis \((21, 22)\) although serum levels are likely less important than local inflammatory signals in the vessel wall. In fact, direct examination of vascular inflammation by

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**Table 1. Serum markers of cardiovascular risk after 6 weeks on diets**

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>SC</th>
<th>WD</th>
<th>LCHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>358.7 ± 28.3</td>
<td>1470 ± 170.7*</td>
<td>1408 ± 251.2*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>108.0 ± 6.9</td>
<td>95 ± 27.8</td>
<td>155 ± 60.7</td>
</tr>
<tr>
<td>Nonesterified fatty acids (mEq/dL)</td>
<td>0.55 ± 0.16</td>
<td>0.86 ± 0.16</td>
<td>1.40 ± 0.26**</td>
</tr>
<tr>
<td>Glucose (fasting; mg/dL)</td>
<td>136 ± 6.9</td>
<td>185 ± 20.5</td>
<td>142 ± 17.7</td>
</tr>
<tr>
<td>Glucose (nonfasting; mg/dL)</td>
<td>NA</td>
<td>406.9 ± 58.9</td>
<td>274.2 ± 51.0**</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>307.5 ± 322.2</td>
<td>432.9 ± 477.9</td>
<td>308.5 ± 205.4</td>
</tr>
</tbody>
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*, \( P < 0.05 \) compared with SC control. **, \( P < 0.05 \) compared with WD.
quantitation of leukocyte accumulation of atherosclerotic plaque in WD- and LCHP-fed mice revealed no differences in the percentages of monocytes or helper T cells in these lesions (Fig. 2F).

**Influence of LCHP Diets on Endothelial Progenitor Cells.** Because neither alterations in major serum lipid components, inflammation nor oxidative stress appeared to account for the increased atherosclerosis seen in LCHP-fed mice, we examined dietary effects on EPCs, a cellular population that integrates into vessels during atherogenesis (23). In clinical studies, circulating EPCs correlate with vascular function (8), and a decrease in the EPC number also independently predicts cardiovascular risk (7). Flow cytometric analyses of peripheral blood mononuclear cells demonstrated a 82–86% decrease in the EPC-containing compartment (Flk1+, Sca1+) in LCHP-fed mice compared with either WD- or SC-fed mice, suggesting that circulating EPC numbers are reduced by a LCHP diet (0.055% and 0.29% vs. 0.39% respectively for LCHP, WD, and SC-fed mice, P = 0.01 for LCHP vs. WD, P = 0.0002 for LCHP vs. SC; Fig. 3A). Circulating EPCs may originate in the bone marrow (24), so we next examined whether the functional endothelial progenitor capacity of bone marrow was similarly impaired. We quantified EPC colony forming units (CFU-EPC) in ApoE−/− mice on the three diets. Whole bone marrow was cultured on a semisolid substrate in the presence of vascular endothelial growth factor (VEGF) and positive colonies identified after 7 days by morphology, acetylated LDL uptake, and Ulex europeaus lectin binding (25). After 6 weeks on the diets, CFU-EPC were decreased by >45% in bone marrow from LCHP-fed ApoE−/− mice compared with that from either WD- or SC-fed mice (77 ± 23 vs. 143 ± 15 vs. 141 ± 24, respectively, P ≤ 0.005 for both; Fig. 3B, Left). Interestingly, a comparable diet-induced decrease was seen in the parental strain (C57BL/6) despite wild-type ApoE alleles, suggesting the dramatic hypercholesterolemia seen only in ApoE−/− mice is not required for this effect (Fig. 3B, Right).

**LCHP Diet Reduces Phosphorylated Akt in EPCs.** To better understand how the LCHP affected EPCs we examined phosphorylation (activation) of the serine-threonine kinase, Akt, a metabolic signaling molecule downstream of the insulin receptor previously shown to regulate EPC mobilization, survival, and proliferation (28–30). Given the extremely low prevalence (0.01–0.03%) of EPC in bone marrow, we used intracellular flow cytometry to assess Akt activation in bone marrow EPCs on a cell-by-cell basis. C57BL/6 mice were maintained on the study diets for 4 weeks before whole bone marrow was harvested and analyzed for total hematopoietic lineage markers (lin), Flk-1 (Flk) expression, and intracellular phosphorylated Akt (Fig. 4A). Lineage-negative, Flk1+ (lin−Flk1+) bone marrow cells from mice on the LCHP diet had significantly less phosphorylated Akt than did these cells from either SC-fed or WD-fed mice. Control populations of lin−Flk−, lin−Flk−, or lin+Flk+ cells from the same animals generally showed no Akt phosphorylation at Ser-473 and this was not affected by the diets (Fig. 4A). Thus, in addition to increasing atherosclerosis in ApoE−/− mice, the LCHP diet impaired neovascularization in wild-type mice, consistent with an effect on EPCs and suggesting broader relevance to these observations beyond the ApoE−/− model of atherogenesis.
interested to examine if differences in serum VEGF levels could account for the differences in circulating EPCs. Serum VEGF levels were similar between SC-fed and WD-fed mice, but higher in LCHP-fed mice, when compared with SC alone (Fig. 4B), suggesting that a decrease in chemoattractant signaling by VEGF is not responsible for the decrease in circulating EPCs in the LCHP mice.

Discussion

Our data demonstrate that atherogenesis and neovascularization can be modulated through alterations in macronutrients other than fat and cholesterol. Because our LCHP diet simultaneously decreases carbohydrates while increasing protein content, it does not allow us to ascribe the effects seen to only one of these alterations. However, these balanced macronutrient changes are necessary to maintain an isocaloric diet, while avoiding alterations in fat content. Whether similar effects would be seen with the more severe limitation of carbohydrate (0.76% wt/wt carbohydrate) in the ketogenic diet recently described (12), where FGF21 expression is induced, is not clear. However, the macronutrient changes in the LCHP diet used here mimic the diets commonly used in humans, where reduced carbohydrate intake is generally accompanied by increased protein and fat intake. Interestingly, multiple results in mice on the LCHP diet paralleled those reported in clinical trials including reduced weight gain without significant changes in serum lipids or other markers.

Fig. 3. Dietary effects on endothelial progenitor cells and neovascularization. (A) Peripheral blood leukocytes positive for Sca-1 (FITC) and Flk-1 (PE) antigens by flow cytometry at 6 weeks on diets. Representative dot-plots from FACS data are shown. Quantitation of the absolute percentage of Sca-1/Flk-1 cells in the circulating leukocyte compartment (peripheral blood leukocytes; PBLs) for four independent experiments is shown in graph below. n = 7, WD = 9, LCHP = 10; results shown as mean ± SD; two-way comparisons by Student’s t test. (B) Bone marrow-derived CFU-EPC after 6 weeks on diets. Left, ApoE−/− mice on diets; Right, C57Bl/6 mice on diets. n for ApoE−/− mice, SC = 4, WD = 7, LCHP = 7; n for ApoE+/− mice, SC = 3, WD = 4, LCHP = 4. Results shown as mean ± SD; two-group comparisons by Student’s t test. (C) Recovery of blood flow after unilateral femoral artery ligation was measured by laser doppler flux over the 28 days after surgery. Lower shows representative flux scans for mice on the WD and LCHP 28 days after surgery (red, high flux; blue, low flux). Left leg, surgical limb; right leg, non-operated control limb. Upper displays cumulative quantitative data expressed as a ratio of flux in the ischemic limb over that in the nonischemic limb (mean ± SEM; # P < 0.001 for WD (■) compared with LCHP (●) using one way ANOVA; n: WD = 20, LCHP = 21). Results shown as mean ± SE.

Fig. 4. LCHP diet reduces Akt phosphorylation in bone marrow EPCs. (A) Flow cytometric analyses of lineage-negative and Flk1-positive (lin− Flk−) whole bone marrow stained for intracellular phospho-Akt (Ser-473) (FITC, green). Control cells are lin− Flk− (purple), lin− Flk− (green) or lin− Flk− (orange) cells from each mouse. Dot plot shows representative distribution of bone marrow cells stained for lineage markers (APC) and Flk-1 (PE). Bar graph shows quantitation of cells in the FITC-channel histogram P3 gate; n = 4 for each cohort. Results shown as mean ± SD. (B) Serum levels of VEGF quantitated by ELISA show increased VEGF expression in LCHP diets compared with SC, but not WD diets. n = 8 for each cohort. *, P < 0.05 compared with SC control. Results shown as mean ± SD; two-way comparisons performed with Student’s t test.
of cardiovascular risk (10, 11), further reinforcing the potential relevance of this model.

Exacerbated atherosclerosis occurred on the LCHP diet independent of significant alterations in traditional atherogenic serum lipids, serum inflammatory markers and histological indicators of inflammatory infiltration. We did detect a significant increase in serum NEFA levels on the LCHP diets (Table 1) but this was not correlated to an increase in serum measures of inflammation. Importantly, there was no evidence of increased leukocyte infiltration in plaques from mice on the LCHP diet. We did not detect a significant difference in either circulating oxLDL or tissue markers of oxidative stress. Together these data suggest that neither an increase in the inciting signals nor in the inflammatory cascade are responsible for the increased atherosclerosis seen on the LCHP diet when compared with the similarly high-fat WD.

We hypothesized that an impaired ability to restore vascular function could accelerate atherosclerosis despite comparable injury and inflammation. Whereas endothelial progenitor cells have been postulated to play a protective role in the adult vasculature, their precise roles remain incompletely defined. Targeted application of EPCs to areas of experimental endothelial injury improves reendothelialization and in vitro measures of endothelial function (5, 32–34), suggesting that these cells can enhance endothelial recovery from injury. EPCs can also be shown to integrate into the adult endothelium (35, 36). However, the frequency of integration appears low, raising the possibility that EPCs could mediate beneficial but indirect effects on existing endothelial cells rather than directly contributing to the generation of new endothelial cells. In our diet-induced atherosclerotic model, we found that a LCHP diet induced a substantial decrease in the number of endothelial progenitors, both in the bone marrow and the peripheral blood. This effect did not appear to be dependent on the absence of the ApoE gene, as wild-type C57BL/6 mice also showed fewer bone marrow-derived CFUs and impaired functional neovascularization after hindlimb ischemia. We also found a reduction in phosphorylation of Akt specifically in the bone marrow EPC-compartment from these mice on the LCHP diet. Interestingly, the differential regulation of Akt phosphorylation occurs only in lineage-negative Flik-positive cells (but not lin− Flk−, lin− Flk1 or lin− Flk− populations), suggesting that Akt inhibition is a specific effect of these diets on EPCs. Previous work suggests Akt activation is important in EPC mobilization, proliferation, and survival (29–31). The reduction in peripheral EPCs also occurred despite increased serum VEGF levels, which demonstrates that the observed decrease in EPC Akt phosphorylation is not secondary to reduced VEGF, an important stimulus for Akt activation in endothelial lineages.

Our data are consistent with a model where atherogenesis may reflect a balance between noxious stimuli (e.g., hyperlipidemia, oxidative stress, inflammation) and homeostatic mechanisms to restore vascular function (possibly including EPCs). Thus, atherosclerosis could be increased in hyperlipidemic states (WD and LCHP, compared with SC controls) by the increase in atherogenic stimuli, but could also be exacerbated by a defect in restorative capacity despite similar exposure to noxious stimuli (e.g., LCHP compared with WD). The observed reduction in EPCs on the LCHP diet, suggests these cells could play a role in this context. However, we cannot exclude the possibility that EPCs are themselves merely a marker, rather than causally related to these phenotypes. Adoptive transfer of EPCs could theoretically address this question but is hampered by the low frequency of these cells (0.01–0.03% of lineage-negative bone marrow cells) and the high likelihood of simultaneously introducing proinflammatory cell populations that are in vast predominance in the bone marrow. Such experiments would be facilitated by improved and more specific markers for EPCs. Nevertheless, the observation that the LCHP increases atherosclerosis and appears to do so independent of alterations in traditional risk factors underscores our incomplete understanding of atherogenesis and should provide a useful model for investigating these alternative mechanisms further.

Aortic Analysis. Mice were killed and aortae were dissected from the aortic arch to the iliac bifurcation. Adventitial fat was removed and the aorta splayed open from the proximal arch to the iliac bifurcation. The splayed aortae were pinned to a wax board, fixed with 80% ethanol and stained with Oil Red O. Aortae were photographed and images analyzed with IP Lab 3.0 software. The aortic root was flash-frozen in OCT media (TissueTek). Cryotomed slides were fixed in absolute propylene glycol, stained with Oil Red O and counterstained with Harris’s hematoxylin. Sections that demonstrated all three leaflets of the aortic valve were selected; planimetric analysis of each section using IP lab 3.0 software allowed quantitation of the total area of plaque in each section. A total of three sections per aorta were measured and averaged. Sections were also fixed in acetone and stained for F4/80 and CD4 antigens using a HRP-colorimetric developer (BD Biosciences). Percentage area stained positive for F4/80 was quantitated using IP lab 3.0 software and the baseline set using the WD-fed mouse aortic root as a positive control. At least 4 sections per aorta were analyzed. For 8-oxoguanine detection, we used a mouse anti-oxo-guanine antibody (Chemicon MAB3560). Sections of aortic root were fixed in absolute propylene glycol and stained with the antibody according to manufacturer’s directions. Secondary staining was performed with rhodamine-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) and counterstained with DAPI. An average of 3 sections were measured per aorta, photographed and the percentage of lesion area positive for 8-oxoguanine staining was quantitated using IP Lab 3.0 software using a colorimetric gating algorithm.

Serum Analysis. For fasting analyses, mice were fasted overnight (16 h). Blood was obtained from right and left ventricular puncture at the time of kill and analyzed by a Critical Chemistry Analyzer Hitachi 917 for total cholesterol, triglycerides, and glucose. Insulin, NEFA, oxidized LDL, VEGF, interleukin-6, and monocyte-chemotacticant-1 levels were measured using ELISAs from kits, per manufacturers’ directions (Insulin, Crystal Chem; NEFA, Wako chemicals; oxidized LDL, Merckodia; IL-6 and MCP-1, BD Biosciences; VEGF, Calbiochem). The distribution of cholesterol among the lipoprotein size classes was analyzed by fast protein liquid chromatography (FPLC). Briefly, 250 μL pooled plasma (n = 3) was filtered (0.45-μm filter) and resolved by size exclusion chromatography using a Superose 6 10/300 GL column (Amersham Biosciences). The cholesterol content of fractions (0.5 mL) was determined enzymatically.
Colonies were plated per well in semisolid methylcellulose media in the femoral artery with subsequent severance of the femoral artery between the ligatures. Immediately after surgery, mice were imaged on a Moor 785 nm near-infrared Laser Doppler Imager-2. At days 2, 4, 7, 10, 15, and 28 after surgery, mice were subjected to repeated scans on the LDI-2. Mice remain on study diets throughout the postoperative period.

Statistics. Two-tailed Student’s t tests were used for comparisons of two variables. One-way ANOVA with repeated measures was used for analysis of weight gain in mice on diets. Statistical calculations were performed on Stata 10.0 software. All data presented are shown as means ± SD except for the flow recovery from HLI, which is shown as mean ± SEM.

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