Dietary Cholecalciferol and Calcium Levels in a Western-Style Defined Rodent Diet Alter Energy Metabolism and Inflammatory Responses in Mice1,2

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Abstract

Male and female C57Bl6 mice were fed a control AIN76A diet, a new Western-style diet (NWD1) reflecting dietary patterns linked to elevated colon cancer incidence (higher fat, lower cholecalciferol, calcium, methyl donors, fiber), or NWD1 with elevated cholecalciferol and calcium (NWD2) from weaning. After 24 wk, serum 25-hydroxyvitamin D [25(OH)D] decreased by >80% in the NWD1 group compared with controls, but with no alteration in serum calcium or bone mineral density. The decreased serum 25(OH)D was prevented in the NWD2 group. After 32 wk, the NWD1 group compared with controls reduced overall energy expenditure by 15% without altering food consumption or physical activity and induced glucose intolerance, phenotypes associated with metabolic syndrome. These responses were unexpectedly exacerbated in the NWD2 group, further shifting mice toward greater fatty acid storage rather than oxidation compared with both control and NWD1 groups, but there was no change in physical activity, causing significant weight gain due to increased fat mass. The NWD1 group also exhibited inflammatory responses compared with controls, including macrophage-associated crown-like structures in epididymal adipose tissue and increased serum concentrations of the proinflammatory cytokine IL-1β, and of its targets, MCP-1 and Rantes, which were prevented or greatly mitigated in the NWD2 group. However, there was also elevated lipid storage in the liver and steatosis not seen in the control and NWD1 groups. Thus, elevating cholecalciferol and calcium in a Western-style diet can reduce inflammation associated with risk for colon tumor development, but interaction of nutrients in this diet can compromise liver function when fed long term. J. Nutr. 142: 859–865, 2012.

Introduction

Newmark, Lipkin, and colleagues (1–4) designed defined Western-style rodent diets based on the formulation of AIN-76A to reflect the levels of intake, on a nutrient density basis, of a number of nutritional factors in the diet of large segments of the population in Western, or developed, countries. The new Western-style diet (NWD1)7 described by these investigators includes higher fat and phosphate than in AIN76A (4) and lower levels of cholecalciferol and calcium, donors to the single carbon pool (folate, choline, and methionine), and fiber. Fat in the diet, from corn oil, was elevated to provide 20% of total energy, which is lower than the levels of $40\%$ in high-fat diets that are commonly used in rodent models of obesity (5). How this level of fat, especially in the context of the levels of other components in the diet, alters metabolic variables has not been determined. Further, the NWD1 stimulates macrophage and T cell infiltration into the intestinal mucosa, suggesting an inflammatory response to the diet. However, this response to the diet has not been well characterized, nor has its modulation by cholecalciferol and calcium, 2 components that have been reported to have effects on inflammation in a human clinical trial (6) and which are varied in the Western-style diets.

These issues were investigated, demonstrating a profound impact of the Western-style diet on patterns of nutrient...
metabolism and energy utilization and tissue-specific and systemic inflammation, all of which are modulated by cholecalciferol and calcium levels in the diet. The data suggest that nutrient interactions determine patterns of response of these variables in mice. Further, some of the variables altered by the NWD1 may underlie shorter term effects of the NWD1 in stimulating tumor development in genetic models of tumor initiation (7,8) and longer term effects of the diet in initiating tumor development (4,9).

Experimental Procedures

Mice. All studies were approved by and performed in compliance with guidelines of the Yeshiva University Institutional Animal Care and Use Committee. C57Bl6 mice were obtained from Jackson Laboratories at 3 wk of age, housed in a barrier facility at the Albert Einstein College of Medicine, and randomized to 3 diets (Research Diets). The diets, fed ad libitum for up to 12 mo, were formulated on the basis of nutrient density. Diets have been described in detail (2,9,10) and are shown in Table 1. In the AIN76A control diet, fat content (corn oil) was 50 g/kg (i.e., 5% by weight) and cholecalciferol and calcium contents were 25 µg/kg (the equivalent of 1 IU/g) and 5 g/kg, respectively. Fat was elevated in the NWD1 to 200 g/kg (20% by weight) and dietary cholecalciferol and calcium lowered to 2.7 µg/kg and 0.5 g/kg, respectively. Folic acid, methionine, and choline, all donors to the single carbon pool, were also reduced (4). In the new Western-style diet with elevated cholecalciferol and calcium (NWD2), the elevated dietary fat and reduced levels of single carbon donors were held at the levels in the NWD1, but cholecalciferol and calcium levels were supplemented to 57 µg/kg and 7 mg/g, respectively. Thus, levels in the NWD1 were equivalent to 200 IU cholecalciferol and 220 mg calcium in a 2000-kcal/d human diet and were elevated in NWD2 to the equivalent human daily intake of 1000 IU cholecalciferol and 3000 mg calcium (4).

Bone micro computed tomography. Micro computed tomography (MicroCT) analyses were performed in the Yale Core Center for Musculoskeletal Disorders microCT Unit. Femur and vertebral morphometry were quantified using cone beam microfocus X-ray computed tomography (µCT35; Scanco Medical). Samples were scanned in ethanol and serial tomographic images were acquired at 55 kV, with an integration time of 500 ms and isometric voxel size of 6 µm. Segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter (support = 1; 3 × 3 × 3 voxel window; σ = 0.8) to reduce noise, applying density thresholds of 250 and 420 for the trabecular and cortical compartments of the femur, respectively. Volumetric regions for trabecular analysis were selected within the endosteal borders of the distal femoral metaphysis to include the secondary spongiosa located 1 mm from the growth plate and extending 1 mm proximally, or from within the cortical shell of the third lumbar vertebral body. Cortical morphometry was quantified and averaged volumetrically through 233 serial cross-sections (1.4 mm) centered on the diaphyseal midpoint between proximal and distal growth plates.

TABLE 1 Diet compositions1

<table>
<thead>
<tr>
<th>Component</th>
<th>AIN76A</th>
<th>NWD1</th>
<th>NWD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (corn oil), g/kg</td>
<td>50.0</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Ca, g/kg</td>
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<td>0.5</td>
<td>7.0</td>
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<tr>
<td>Cholecalciferol, µg/kg</td>
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<td>2.7</td>
<td>57.0</td>
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<tr>
<td>P, g/kg</td>
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<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
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<td>0.2</td>
</tr>
<tr>
<td>DL-methionine, g/kg</td>
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<td>0.0</td>
</tr>
<tr>
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<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
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<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>L-cysteine, g/kg</td>
<td>2.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

1 NWD1 and NWD2 are based on AIN76A. Rationale and formulations are described in detail in (4). The components shown are only those that are altered among the diets used in this report. Adapted with permission from (4). NWD, new Western-style diet.

FIGURE 1 Serum 25(OH)D (A), PTH (B) and calcium concentrations (C) and spine trabecular (D), femur trabecular (E), and femur cortical BMD (F) of mice fed the AIN76A, NWD1, or NWD2 diet for 24 wk. Data are mean ± SEM, n = 3. Labeled means without a common letter differ, P < 0.05. [1 mg/mL 25(OH)D = 0.4 nmol/L; 1 pg/mL PTH = 0.11 pmol/L; 1 mg/dL calcium = 0.25 nmol/L.] Data were analyzed by ANOVA. BMD, bone mineral density; NWD, new Western-style diet; 25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone.
Glucose tolerance test. Mice were fed deprived for 12 h and i.p. injected with 1 g/kg of D-glucose in isotonic saline. Blood was collected from the lateral tail vein prior to and at 15, 30, 45, 60, 90, and 120 min after injection. Glucose was measured using a Precision Q.I.D. monitoring system (MediSense, Abbott Laboratories). A similar protocol was used for measuring the response of plasma insulin to the glucose load using an ELISA kit (Mercodia), except that blood (30 μL) was collected at 0, 30, and 60 min after injection for insulin determination.

Analysis of plasma and serum. Mice were anesthetized with pentobarbital (50 mg/kg), and killed by cervical dislocation. To obtain plasma, blood was centrifuged 10 min at 12,000 g at 4°C and plasma was collected and stored at −80°C. Basal insulin levels, TG, and nonesterified fatty acids (NEFA) were quantified using an insulin ELISA kit (Mercodia), an infinity kit (Thermo Scientific), and a NEFA kit (Wako), respectively. To obtain serum, whole blood was allowed to clot at room temperature for 10 min, centrifuged at 12,000 g at 4°C for 10 min, the serum supernatant was aspirated and centrifuged similarly a second time for 8 min, and the supernatant was stored at −80°C until analysis. Calcium was measured using a colorimetric assay (Genzyme Diagnostics), 25-hydroxyvitamin D [25(OH)D] was measured by RIA (DiaSorin), and intact parathyroid hormone (PTH) was measured by ELISA (Immutopics).

**Homeostatic model assessment.** Insulin resistance was determined using the equation: HOMA-IR = fasting insulin (µU/mL) × fasting glucose (mg/dL)/405, where glucose and insulin are given in mg/dL and µU/mL, respectively.

**Whole body indirect calorimetry.** Oxygen and CO2 consumption were simultaneously determined using an Oxymax open-circuit indirect calorimetry 10-cage system (Columbus Instruments). Mice were allowed to acclimatize for a complete light and dark cycle (24 h). Subsequent measurements were taken for 48 h (2 consecutive light and dark cycles). Instrument settings were: gas flow rate = 0.6 L/min, sample flow rate = 0.5 L/min, settle time = 55 s, measure time = 5 s. Data were normalized by body weight. Energy expenditure was calculated using the equation of Weir: energy expenditure [kcal/(kg·h)] = (3.815 × VO2] + (1.232 × VCO2) where VO2 is oxygen consumption and VCO2 is CO2 consumption.

**Total body mass and MRI.** To determine fat and lean mass, mice were placed in a clear plastic holder without anesthesia or sedation and inserted into the EchoMRI-3-in-1 System from Echo Medical Systems.

**Tissue lipid content.** Mice were feed deprived overnight, anesthetized with pentobarbital (50 mg/kg), and killed by cervical dislocation. Tissues were collected into ice-cold PBS and homogenized using a Bullet Blender (Next Advance) according to the manufacturer’s protocol. Aliquots were taken for protein quantification using the BCA Protein Assay (Thermo Scientific) and for lipid content determination using Folch extraction as described (11). TG was determined using the infinity kit (Thermo Scientific). Results were normalized by protein content.

**Cytokines.** Cytokine levels were determined using a Luminex assay from Millipore on sera from feed-deprived mice.

**Immunohistochemistry.** Immunohistochemistry was performed using the Biomodule IHC staining kit from Invitrogen. Ten-μm serial sections were dewaxed and epitope retrieval was performed by immersing the slides into a citrate buffer at 100°C for 20 min. Slides were incubated with primary antibody raised against the macrophage-specific marker F4/80. Primary antibody was omitted from negative controls. Sections were counterstained with hematoxylin.

**Western blot.** Mice were anesthetized with pentobarbital (50 mg/kg) and killed by cervical dislocation after overnight feed deprivation. Tissues, rapidly freeze-clamped in liquid nitrogen, were stored at −80°C. Protein preparation and blotting were as described below. Tissues were homogenized in buffer [50 mmol/L Tris (pH 7.4), 1% glycerol, 1% Triton X-10] supplemented with protease inhibitors (Complete mini, Roche Pharmaceuticals) using a Bullet Blender, as above. Homogenates were centrifuged 30 min at 21,000 × g at 4°C, supernatants collected, and protein concentration determined with the BCA Protein Assay (Thermo Scientific).

**Protein samples (40–80 μg) were separated on 8 or 10% reducing polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membranes. Immunoblots were blocked with 2% milk and 3% BSA in Tris-buffered saline for 60 min at room temperature and incubated overnight at 4°C with the indicated antibodies (Cell Signaling) in Tris-buffered saline and 0.05% Tween 20 containing 1% BSA. Blots were washed in the same buffer and incubated with HRP-conjugated secondary antibodies (1:30,000) for 30 min at room temperature. Membranes were washed in the same buffer and antigen-antibody complexes visualized by chemiluminescence using an ECL kit (Pierce). The density of the bands was quantified using the Quantity One 1-D Analysis Software from Bio-Rad.

**Statistical analyses.** Data were analyzed by 1-way ANOVA followed by post hoc Tukey’s Multiple Comparisons Tests. Differences were considered significant at P < 0.05. All data are presented as mean ± SEM.
Results

**Serum 25(OH)D, calcium, and bone mineral density.** Serum 25(OH)D was much lower in mice consuming the NWD1 compared to mice fed the control AIN76A diet or NWD2 (Fig. 1A). However, even though dietary calcium content was decreased in the NWD1, serum levels of calcium remained unchanged among all groups (Fig 1C). These results may be due to the reduced intake of cholecalciferol in the NWD1 group and resultant secondary hyperparathyroidism due to impaired calcium absorption and increased conversion of 25(OH)D to its active metabolite, 1,25-dihydroxyvitamin D, to promote intestinal calcium absorption and maintain serum calcium levels. Consistent with this, PTH levels did not significantly differ in mice consuming the NWD1 and NWD2 compared to the AIN76A group (P = 0.2) (Fig. 1B). PTH increases bone resorption and can lead to decreased bone mineral density (BMD) and osteoporosis. However, evaluation of BMD by microCT showed that neither trabecular nor cortical BMD was altered among the mice fed the different diets for 24 wk (Fig. 1D–F) and no profound skeletal defects were observed (not shown). In addition, elevating cholecalciferol and calcium in NWD2 had a significant (P < 0.05) effect on elevating the femur cortical BMD compared to mice fed the AIN76A or NWD1 (Fig. 1F). Although this increase appeared to be coincident with higher PTH, the PTH levels did not significantly differ among the 3 groups. Thus, the reduction in dietary cholecalciferol and calcium in the NWD1 did not cause well-recognized physiological consequences of deficiency of cholecalciferol and calcium in these mice.

**Dietary effects on metabolic variables.** The AIN76A and NWD1 groups gained weight at similar rates over the course of 1 y, whereas the NWD2 group showed greater weight gain (Fig. 2A). For example, after 32 wk, the body mass of the NWD2 group was >40% higher than that of the AIN76A and NWD1 groups (P < 0.05) (Fig. 2B). Although lean mass increased in the NWD2 group compared to the AIN76A and NWD1 groups, (Fig. 2B), this was modest, and the weight gain of the NWD2-fed
mice mostly reflected a dramatic doubling in fat mass ($P < 0.05$) (Fig. 2C).

The effect of diet on substrate utilization was determined in metabolic chambers after 32 wk of receiving the diets. The respiratory exchange ratio (RER) in the NWD1 mice was lower than that in the AIN76A mice, demonstrating a shift from carbohydrate to fatty acid utilization in the NWD1 mice (Fig. 3A) that was consistent with the higher fat content of this diet (well established to reduce RER). The RER in the NWD2 group was further decreased from that of the NWD1 group, demonstrating that cholecalciferol and calcium levels further shifted mice toward even greater relative fat utilization (Fig. 3A). Moreover, overall energy expenditure in the NWD2 mice was lower (Fig. 3B) than in the NWD1 mice without substantial change in physical activity ($P = 0.08$) (Fig. 3D). Therefore, although the NWD2 mice utilized lipids more efficiently, storage rather than oxidation was enhanced. This is consistent with the increased fat mass (Fig. 2B), lower oxygen consumption levels (Fig. 3C), and lower concentrations of plasma TG and NEFA (Fig. 3E, F) in mice fed the NWD2.

Fasting plasma glucose and insulin concentrations did not differ between the AIN76A and NWD1 groups (Fig. 4A, B). However, HOMA-IR, an index of insulin sensitivity, was 16% greater in NWD1 (8.58 ± 0.04) compared to AIN76A (7.39 ± 0.04) mice ($P < 0.05$). Consistent with this, glucose clearance was also moderately impaired in the NWD1 compared to AIN76A mice (Fig. 4C). In contrast to these modest effects, the NWD2 mice fed higher cholecalciferol and calcium but the same fat levels as the NWD1 mice had 40% higher glucose and 225% higher insulin concentrations (Fig. 4A, B). In addition, glucose clearance was more severely impaired than with the NWD1 (Fig. 4C), and HOMA-IR increased 3.2-fold compared to AIN76A mice (24.0 ± 0.71 compared to 7.39 ± 0.04; $P < 0.01$). These
findings all indicate that elevating cholecalciferol and calcium in the context of higher dietary fat exacerbated, rather than prevented, metabolic alterations.

**Dietary effects on inflammation.** NWD1 mice had a >100% higher colon TG content (Fig. 5A), which can promote an inflammatory response. In this regard, the proinflammatory cytokines IL-1β and MCP-1 (also termed CCL2) were elevated in the plasma of NWD1 mice. MCP-1 is a target of IL-1β. A second target, Rantes, also tended to be elevated in NWD1 mice (P = 0.07). Elevating dietary cholecalciferol and calcium in NWD2 mice prevented the increase in IL-1β, significantly reduced the increase in MCP-1, and also reduced the increase in Rantes (Fig. 5B–D). Consistent with this greater systemic inflammatory response in the NWD1 mice and its mitigation by elevating dietary cholecalciferol and calcium, the mucosa of mice fed the NWD1 exhibited inflammatory infiltrates, including elevated macrophages (12). Moreover, adipocytes of the NWD1-fed mice were surrounded by brown-stained, F4/80-positive, macrophage crown-like structures, demonstrating increased tissue inflammation, which was prevented by feeding NWD2 (Fig. 5E).

**Hepatic steatosis.** Mice fed the 3 different diets all had equivalent levels of phosphorylation of AKT on serine 473 in adipose tissue (Fig. 5F). This suggests that despite a decrease in insulin sensitivity in the adipose tissue of the NWD2 mice, this could not account for the difference in glucose clearance between the NWD1 and NWD2 groups. However, there was profoundly altered liver morphology in the NWD2 mice, indicated by numerous lipid vacuoles (Fig. 6A) and a 350% increase in liver TG content (Fig. 6B) compared to the AIN76A- and NWD1-fed mice. This was associated with a 60% decrease in Akt phosphorylation levels in the liver, suggesting that the insulin pathway was severely altered in the liver of the NWD2-fed mice (Fig. 6C).

**Discussion**

The Western-style rodent diet adjusts the content of a number of nutrients to reflect their level of consumption that characterizes large segments of the population in developed countries and therefore to levels that may be linked to diseases prevalent in these areas. The data presented here demonstrate that the levels of cholecalciferol and calcium appear to be major determinants of the profile of energy use and fat disposition in the context of the higher fat and lower methyl donors in the diets. The higher fat in both the NWD1 and NWD2 leads initially to more rapid weight gain, increased fat utilization, and impairment of glucose tolerance. However, in the presence of higher dietary cholecalciferol and calcium, the mice gain significantly more weight, show an even greater shift toward fat utilization, and develop a more significant impairment of glucose tolerance. This is associated with a shift from excess lipids in the colon and adipose tissue to lipid storage in the liver.

Feeding the NWD1 is also associated with development of macrophage associated crown-like structures in the adipose tissue and elevation of proinflammatory cytokines in the serum (Fig. 5) as well as an inflammatory cell infiltration into the intestinal mucosa (12), which were prevented by the elevation of cholecalciferol and calcium in the NWD2. This is important, because local and/or systemic inflammation has been linked to chronic diseases in developed countries, such as cancer, obesity, and diabetes (13). Indeed, long-term feeding of the NWD1 causes the development of colon and small intestinal tumors in wild-type mice (4,9), which was prevented by elevating cholecalciferol and calcium in the NWD2, thus linking the inflammatory responses to eventual intestinal tumor development. This is consistent with the protumorigenic role of macrophages in other systems (14). More specifically, Klampfer et al. (15–17) dissected mechanisms of crosstalk between macrophages and colon tumor cells, mediated by synthesis and secretion of IL-1β from the macrophages, one of the proinflammatory cytokines modulated in the serum by diet. This stimulates Wnt signaling, Snail expression, and Trail resistance in the colonic tumor cells, all of which are prevented by 1,25 dihydroxycholecalciferol (CCL2) inhibition of IL-1β secretion from the macrophages. Moreover, CCL2 (MCP-1) is another cytokine that is elevated in serum in the NWD1 mice, which is prevented by elevating dietary cholecalciferol and calcium levels (Fig. 5) and has recently been shown to be effective in recruiting macrophages to sites of mammary tumor cell metastasis (18).

Thus, we hypothesize that similar to mechanisms that promote the ability of cells to metastasize to distant sites, the probability that initiated cells at the primary site can progress to tumors is modulated through effects of cholecalciferol and/or calcium on cytokine-mediated pathways on the microenvironment of the mucosa, with higher levels of these nutrients limiting inflammation and tumor development. However, elevated cholecalciferol and calcium, in the context of the other components of the Western-style diet, channeled excess lipid to the liver. Although this reduced both intestinal tissue and systemic inflammation, it ultimately led to impairment of liver function. This reflects data in the initial report on these diets that both NWD1 and NWD2 caused liver enlargement and hepatic nodules that contained eosinophilic, basophilic, and vacuolated cells, especially in mice fed the NWD2 (4).

In summary, dietary approaches to alter disease incidence are complicated by competing effects of combinations of nutrients on different organ systems. We have demonstrated in mice that elevating cholecalciferol and calcium in the context of the levels of other nutrients limits the inflammatory response and thus perhaps the longer term potential of colon tumor development, but at the expense of causing hepatic steatosis and long-term potential of tumor promotion in that organ. It is not clear whether these same effects will be seen in humans. For example, epidemiological studies have clearly demonstrated a very strong association of higher cholecalciferol levels with lower probability of human colon tumor development (6,19–24), but intervention studies significantly increasing vitamin D have not been carried out at sufficiently high supplemental levels or for sufficiently long periods to definitively determine the effects on organ-specific physiological or pro-antitumorigenic changes (25–27). The dramatic interaction of nutrient-level intake over a considerable portion of the animals’ life-span demonstrated here emphasizes the importance of monitoring the effects of dietary intervention over very long periods to understand both the profound benefits and complications of adjusting long-term dietary patterns.

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experiments; C.C.B. and E.G.-S. analyzed the data; C.C.B., E.G.-S., J.E.P., and L.H.A. wrote the manuscript; and L.H.A. had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited


