Maturation-Dependent Acquired Coronary Structural Alterations and Atherogenesis in the Dahl Sodium-Sensitive Hypertensive Rat

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Background—The Dahl sodium-sensitive hypertensive rat exhibits atherogenic lesions after the initiation of a high-sodium/high-fat diet. This study was designed to gauge the effect of a preadolescent high-fat diet on the postadolescent rate of atherogenesis after supplementation of the diet with sodium.

Methods and Results—Fifty-three Dahl S male rats were assigned to 2 dietary groups for the postweaning to early adolescence period (3 to 12 weeks): 29 to a standard diet (low-fat/low-sodium) and 24 to a high-fat/low-sodium diet. At age 9 weeks (just after puberty), animals from the high-fat group exhibited a relatively diminished density of coronary elastic fibers. There was no evidence of either lipid or monocytic infiltration of the subendothelial space. At age 12 weeks, most or all of the remaining animals in both groups were switched to a high-sodium/high-fat diet and were sampled through the following 8 weeks for the appearance of arterial lipid. After the switch, the high-fat–conditioned animals developed more extensive atherosclerotic pathological lesions more rapidly than their prepubertal standard-diet counterparts. The importance of the animal’s stage of maturation in this effect was underscored by the observation that delaying onset of the high-fat diet to early adolescence resulted in no ultimate difference from the pubertal controls in elastic fiber density.

Conclusions—The maturation-dependent high-fat conditioning of these postweanling rats correlated with an accelerated rate of atherogenesis on the initiation of the high-sodium/high-fat diet, possibly as a direct result of an alteration in arterial elasticity. (Circulation. 2002;106:2486-2490.)

Key Words: coronary disease ■ elasticity ■ hypercholesterolemia ■ hypertension

The atherogenic process might be divided, pragmatically, into 2 phases. The first phase would encompass infiltration and retention of atherogenic lipoproteins (those containing apolipoprotein B) in the subendothelial space. The second phase would encompass the progressive pathology of the atherosclerotic lesion as described by the Stary staging system. In this scheme, the first phase is driven primarily by physicochemical processes (eg, concentration-dependent lipoprotein transport into the arterial wall); the second phase, by cellular processes orchestrated primarily by infiltrating monocytes. The first phase is readily reversible; the second phase, much less so. The oxidation of LDL after it has entered the subendothelial space might be considered a transitional point between the 2 phases. Subendothelial LDL is retained in the filamentous extracellular matrix, where its lipid components are oxidized by secreted oxidative products from arterial wall cells. In a hypertensive environment, however, the infiltration of LDL into the arterial wall is extended in degree and duration. Pressure-driven convection can accentuate LDL accumulation in the arterial wall. Moreover, hypertension is associated with diffuse arterial wall intima-media thickening. Increased arterial wall thickness in turn reduces oxygen transmissibility, producing hypoxia and steep PO2 gradients within the wall and leading to generation of oxygen radicals, which are both directly injurious to tissue and capable of converting trapped LDL to oxidized LDL. In addition, fluid shear stress induces the expression of the endothelial lectin-like oxidized LDL receptor, which could thus increase the accumulation of oxidized LDL in the arterial intima.

Although hypertension may be a primary factor influencing early LDL accumulation in the subendothelial space, there is little information on any role of hyperlipidemia in normotensive individuals, especially the prepubescent, which might influence the severity of the atherogenic process later, should the individual become hypertensive. It is established that the cross-linking of arterial elastin monomers (which provides vessel elasticity) occurs rapidly during childhood, that LDL binds to elastin, and that an increased arterial stiffness occurs in normotensive children (9 to 13 years old).
diagnosed with primary familial class IIA hypercholesterolemia, which is independent of blood pressure. In addition, it has been shown in vitro that elastin readily complexes via hydrophobic interactions with certain long-chain unsaturated fatty acids, resulting in an increased proteolytic sensitivity. Would persistent hyperlipemia beginning before puberty in a normotensive individual genetically at risk for coronary heart disease directly alter arterial structure in a way that would negatively influence the subsequent capability of an older, but now hypertensive, adolescent or adult to clear LDL from the arterial wall—in effect, weaken the artery’s resistance to the atherogenic cascade triggered by hypertension? To address this question, we have been investigating a controlled model of coronary atherogenesis using the sodium-sensitive hypertensive Dahl S rat (Rapp strain). This strain is an inbred line derived from the original Dahl sodium-sensitive hypertensive rat, for which the genetic area responsible for the sodium-sensitive hypertension response has been mapped. Survival of the animals on a high-sodium (8% NaCl) diet is enhanced if they are allowed to reach the age of 12 weeks on a low-sodium (0.4% NaCl) diet. The delay in starting the high-sodium diet results in a less extreme systolic blood pressure and slower development of hypertension, reaching 185 mm Hg systolic blood pressure after another 4 to 8 weeks. If 4-week-old Dahl S male rats are fed a diet high in both sodium and fat for 8 weeks, cholesteryl ester deposited in the aorta is increased by 70% versus rats fed diets low in either sodium or fat or both.

In this controlled study using the Dahl S model, we first quantitatively describe the adverse effect on coronary elastic fiber structure of hyperlipemic conditioning from weaning to puberty under normotensive conditions (high-fat/low-sodium diet). After the switch of such affected animals and their age- and diet-controlled counterparts to a high-sodium/high-fat (HS/HF) diet during early adolescence, we then quantify the rapidity and extent of coronary atherogenic changes as a function of the animal’s preadolescent diet.

Methods

Study Design

Animals and Preadolescent Vascular Conditioning

A total of 53 Dahl S male rats (sodium-sensitive hypertensive, Rapp strain) were received in groups of 2 to 10 from Harlan Sprague Dawley (Indianapolis, Ind) at various ages from just after weaning (age, 26 days) to age 61 days. Animals received after age 26 days had been maintained at Harlan Sprague Dawley on a low-sodium rat chow (0.4% NaCl). Animals were assigned to 1 of 2 postweaning dietary groups, either a standard low-sodium rat chow (n = 29) (group 1) or the high-fat/low-sodium conditioning diet (n = 24) (group 2). Ten animals from each group (subgroup 1P and 2P; “P” for puberty) were euthanized at age 66 days (just after puberty) for histological analysis of right posterior descending (RPD) coronary arteries.

Adolescent HS/HF and Age/Diet Control Subgroups

At age 12 weeks, 12 (now adolescent) animals from group 1 and 14 from group 2 were switched to an HS/HF diet, and the RPD arteries were histologically analyzed at the animal’s natural death or euthanasia up to 8 weeks later (subgroups 1A and 2A; “A” for atherosgenic). The remaining 7 animals from group 1 were used as adolescent controls for the high-fat conditioning diet used for the weanlings (subgroup 1F; “F” for fat). They were switched to the high-fat conditioning diet at age 12 weeks, and their coronary vessels were examined at the end of their individual life spans. All procedures involving the rats were conducted in accordance with institutional guidelines for the care and use of animals.

Test Diets

The standard diet was the natural basal diet 5001 containing 4.5% fat and 0.02% cholesterol but without added NaCl, thus ≈0.4% NaCl (No. 18049, Purina Mills Test Diet). The high-fat diets, both low-sodium (0.4% NaCl) and high-sodium (8.0% NaCl) variations, were modifications of the purified basal diet 5755 containing 10% coconut oil, 1.5% cholesterol, and 0.5% sodium cholate/cholic acid (No. 20378, No. 29163; Purina Mills Test Diet).

Serum Lipid Chemistry

Serum lipid profiles were determined on a Synchron LX20 (Beckman Coulter, Inc). In this system, total cholesterol values are linear to 1000 mg/dL, HDL cholesterol is linear to 135 mg/dL, and triglycerides are linear to 950 mg/dL.

Histological Analysis

Tissue Preparation

The heart (typically 20 mm from apex to base) was immersed in O.C.T. embedding compound (Sciscope Instrument Co), rapidly frozen, and sectioned transversely 5 mm from the apex, at which point ten 10-μm serial sections were cut for histological stains. The frozen sections were adhered to microscope slides and allowed to air-dry, then were stored at −20°C or colder until they were stained.

Histological Stains

Evaluation of the general architecture of the cardiac section and inflammation (if any) was interpreted on sections stained with hematoxylin-eosin, which was performed as one of the research support services of the Diabetes and Endocrinology Core Laboratory of the University of Massachusetts Medical School. The density and organization of arterial elastin was evaluated on sections stained by Miller’s Elastin/Picro-Sirius Red combination stain. In this method, elastin is stained intensely black and collagen is stained red. The density of arterial lipids was measured on sections stained with Oil Red O.

Histomorphometry

Densities of both arterial lipid and elastin were measured on appropriately stained sections by the same general method. An unfiltered color digital microscopic image of the most prominent and exterior RPD vessel was captured, printed on premium inkjet glossy paper, and scanned in gray scale (lipid) or red-filtered (elastin) with a GS-700 Imaging Densitometer/Multi-Analyst program (Bio-Rad Laboratories). Morphometric measurement of the lipid or the elastin strand density of the vessel wall was calculated from the program-integrated optical densities related to the particular stained feature as manually circumscribed on the scanned image.

Specifically, wall elastin density (WED) was calculated from the parameters U1OD (mean optical density of the elastin-stained vessel wall) and U2OD (mean OD of perivascular matrix similar to matrix background of vessel wall), WED = U1OD – U2OD. Thus, wall matrix background was automatically subtracted from each final WED result.

Similarly, wall lipid density (WLD) was calculated from the parameters U1OD (mean optical density of the lipid-stained vessel wall) and U2OD (mean OD of perivascular matrix similar to matrix background of vessel wall), WLD = U1OD – U2OD. A WLD reference range for prepathological lipid accumulation (ie, “first-phase” atherogenesis) was provided by the WLD values for the 6 animals of subgroup 1F in which no arterial wall lipid was apparent by the Oil Red O stain. The upper limit of this range would thus reflect the maximal limit of any possible Oil Red O tinge that might be contributed by passively infiltrated but uncoalesced lipid accumulated from the high-fat diet over an adult lifetime.
By use of power analysis, the t test for difference in means was used to assess subgroup mean differences for the continuous variables of serum total cholesterol and arterial WED (as a function of diet) and subgroup mean differences for arterial WLD (as a function of days on the HS/HF diet). Differences were considered statistically significant at a value of $P < 0.05$.

**Results**

**Prepubertal Diet and Arterial Elastic Fiber Alterations**

As graphically illustrated in Figure 1, animals conditioned on the high-fat diet from weaning to puberty (subgroup 2P) exhibited significantly less dense elastic fibers in the RPD coronary artery (mean WED, 0.19; SD, 0.084; n=10). Subgroup 1P, standard diet to puberty (age, 9 weeks): mean WED, 0.27; SD, 0.051; n=10. Subgroup 1F, standard diet to early adolescence (age, 12 weeks), then high-fat diet for balance of life (4 to 30 weeks): mean WED, 0.28; SD, 0.072; n=7. Subgroup 2P mean WED is significantly different from both subgroups 1P and 1F ($P < 0.05$).

In addition to differences in elastic fiber density, individuals from subgroup 2P were also more likely to exhibit altered interfiber arrangement at puberty, ie, more variance from parallel orientation, than their counterparts fed the standard diet (subgroup 1P: mean WED, 0.27; SD, 0.051) ($P < 0.05$). That this density effect is dependent on the animal’s stage of maturation is underscored by the observation that animals begun on the same high-fat conditioning diet in early adolescence (age, 12 weeks) exhibited no significant difference in elastic fiber density (1F: mean WED, 0.28; SD, 0.072) from the pubertal standard diet controls (subgroup 1P) for their entire life spans (range, 112 to 296 days; mean, 239.7; SD, 70.6).

In addition to differences in elastic fiber density, individuals from subgroup 2P were also more likely to exhibit altered interfiber arrangement at puberty, ie, more variance from parallel orientation, than the standard diet controls (Figure 2B versus 2A). These architectural differences in the elastic fibers remained unchanged into early adolescence (age, 12 weeks) (data not shown). It is important to note that these alterations occurred in the absence of observable infiltration of monocytes or even of pooled lipid (not shown), although the accumulation of lipid below detectable levels cannot be ruled out. Thus, an explanation relying on monocyctic elastase is unlikely.

**Statistical Analysis**

By use of power analysis, the t test for difference in means was used to assess subgroup mean differences for the continuous variables of serum total cholesterol and arterial WED (as a function of diet) and subgroup mean differences for arterial WLD (as a function of days on the HS/HF diet). Differences were considered statistically significant at a value of $P < 0.05$.

**Preadolescent Diet and Rapidity of Adolescent Arterial Lipid Accumulation**

Although no pooled lipid in the coronary arterial walls was observed throughout the period of prepubertal high-fat conditioning, in such animals, lipid rapidly accumulated after the conditioning diet was supplemented with 8% sodium chloride at age 12 weeks (Figure 3). As depicted in Figure 4, 13 of the 14 conditioned animals (subgroup 2A) quickly accumulated pooled arterial lipid during the 8 weeks after the switch to the

**Figure 1.** WED of RPD coronary arteries as a function of diet during different maturation states. Subgroup 1P, standard diet to puberty (age, 9 weeks): mean WED, 0.27; SD, 0.051; n=10. Subgroup 2P, high-fat diet to puberty: mean WED, 0.19; SD, 0.084; n=10. Subgroup 1F, standard diet to early adolescence (age, 12 weeks), then high-fat diet for balance of life (4 to 30 weeks): mean WED, 0.28; SD, 0.072; n=7. Subgroup 2P mean WED is significantly different from both subgroups 1P and 1F ($P < 0.05$).

**Figure 2.** Alterations of arterial elastin architecture in rats at puberty as a function of diet from weaning. A, Subgroup 1P animal (standard diet): WED, 0.22. B, Subgroup 2P animal (high-fat diet): WED, 0.06. Note generally diminished elastic fiber intensity and greater variance from parallel orientation. Miller’s Elastin/Picro-Sirius Red; magnification x200. Scale bar=40 μm.

**Figure 3.** Rapid lipid infiltration into a small RPD vessel wall triggered by dietary sodium. A, Subgroup 2A weanling animal conditioned on high-fat diet until early adolescence and then continued on same diet supplemented with 8% NaCl (HS/HF diet) for next 8 days. WLD was 0.28. B, Animal on same diet plan but after only 1 day on HS/HF diet. WLD was not measured because slide was counterstained to better reveal endothelium. Oil Red O; magnification x200. Scale bar=25 μm.
HS/HF diet (the 1 lipid-negative animal died 1 day after sodium supplementation). In contrast, only 1 of the 13 standard-diet animals (subgroup 1A) accumulated pooled arterial lipid, later during the same 8-week interval. Mean arterial wall lipid (WLD) values were significantly different for the 2 subgroups (2A: mean, 0.28; SD, 0.067; 1A: mean, 0.15; SD, 0.055; \( P < 0.05 \)).

**Serum Lipid Chemistry and Atherosclerotic Histopathology**

Serum lipid profiles (nonfasted) were analyzed for the animals of subgroups 1P and 2P at death (puberty). Although not measured directly, the LDL cholesterol can be assumed to represent the majority of the difference between total and HDL cholesterol values. The mean values for subgroup 1P (standard diet), in mg/dL, were: total cholesterol, 90 (SD, 16); HDL cholesterol, 58 (SD, 7.8); and triglycerides, 274 (SD, 93). In contrast, mean values for the high-fat diet–conditioned subgroup 2P were total cholesterol, 513 (SD, 236); HDL cholesterol, 42 (SD, 28); and triglycerides, 101 (SD, 34). Mean total cholesterol values were thus significantly (>5 times) higher for subgroup 2P (\( P < 0.0005 \)).

It took longer to observe RPD atherogenic changes in animals of subgroup 1A, shifted from the standard to the HS/HF diet at age 12 weeks, than in animals of subgroup 2A, shifted from the high-fat conditioning diet at the same age (Figure 4). As a rule, both arterial lipid and mononcytic pathology, ie, foam cells with or without lumenal monocytes, were observed concurrently. Arterial wall lipid could be demonstrated without mononcytic pathological lesions in only 1 animal of subgroup 1F, an animal that had progressed 30 weeks beyond early adolescence on the high-fat conditioning diet (not shown). All of the animals in subgroup 2A except the one that died within 24 hours of the diet switch demonstrated extensive mononcytic pathological lesions. The RPD arterial disease was observed to advance as far as a stage of severe acentric stenosis (>50% lumenal diameter), intima filled with foam cells and extracellular lipid pools, disrupted smooth muscle, and perivasculary inflammation, features characteristic of stage III atheromatous lesions. Figure 5 displays one such advanced-stage vessel only 10 days after the dietary switch in this animal from subgroup 2A.

**Discussion**

The accumulated data support the scenario outlined in our hypothesis: a high-fat diet from weaning to puberty weakens the arterial wall by depleting and/or altering its elastic fiber organization, and once so weakened, the artery is more susceptible to the higher shear stresses that follow the introduction of dietary sodium, leading to an accelerated pace of lipid infiltration into the vessel wall, perivascular inflammation, and the continuance of the atherogenic cascade. In contrast, animals allowed to mature with the standard diet have comparatively much more elastic arteries at puberty and are much better able to handle the fluid shear stresses that are brought with the introduction of dietary sodium at 12 weeks. Thus, the duration of an HS/HF diet is not nearly as important to the development of atheromas as is the timing of the initiation of a high-fat diet (weaning versus adolescence).

The constitutive impairment of endothelial nitric oxide synthase (eNOS) activity in Dahl S rats might at first also appear to play a role in the development of the atheromatous lesions observed in these animals (diminishment of the • NO sink for free radicals, allowing increased oxidation of LDL). However, hypercholesterolemia enhances eNOS production of superoxide as well as NO, supraoxide reacts with NO to form peroxynitrite, and both superoxide and peroxynitrite...
are powerful oxidants of lipoproteins. Thus, the impairment of eNOS in hypercholesterolemic Dahl S rats would tend to ameliorate this alternative proatherogenic effect, in much the same way as eNOS-deficient mice show reduced fatty-streak formation (versus control mice) under hypercholesterolemic conditions. The mechanism responsible for the elastin reductions in the animals reaching puberty on the high-fat diet is unlikely to involve elastolytic processes, because the reductions occurred before any observable inflammatory cell presence (consistent with a low-sodium diet). Rather, the mechanism may involve inhibition of molecular cross-linking of the elastin monomers through steric hindrance by bound LDL, as might be inferred from the in vitro LDL–elastin binding data reported by Podet et al. The closest clinical analogy to our experimental design would be primary familial class IIA hypercholesterolemia, in which increased carotid artery stiffness (versus controls matched for age and blood pressure) has been found to be independent of blood pressure (n = 30; age, 11.2 ± 2 years). Such children go on to develop echographic evidence of early carotid atherosclerosis later in adolescence. The present study has several limitations. The data on higher mean total serum cholesterol in the pubescent animals maintained on the high-fat diet is admittedly of limited usefulness in drawing correlations with vascular pathological changes without a complete analysis of the transport system. Nevertheless, it was necessary to document the hypercholesterolemia in this model. Also, the lack of blood pressure data here limits the ability to draw quantitative correlations between the magnitude of hypertension and WLD. However, the statistically significant elevation of mean WLD in subgroup 2A over 1A in the post–diet switch period (Figure 4) demonstrates a clear functional consequence of a high-fat preadolescent diet regardless of the specific mean elevation of systolic blood pressure (the indirect effect of the high-sodium diet). Any direct effects of a high-sodium diet on vascular disease would apply to both subgroups. A useful feature of this model is the ability to both set the onset and extend the duration of atherogenesis by delaying the commencement of 8% dietary NaCl to early adolescence (age, 12 weeks). Such a feature allows for a large temporal window to correlate the histopathological sequence of lipid infiltration → foam cell development → vascular occlusion with serum markers for the onset of the earliest (and most reversible) stages of atherosclerosis. Another valuable feature of this model would be the ability to correlate the effects of intervention strategies (dietary and/or pharmacological) on atherogenesis in terms of both clinical laboratory and histopathological staging.

Acknowledgments
This work was supported by the University of Massachusetts Medical School, Worcester, Mass, and by gifts from Dr. Robert P. Griffin.

References
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Circulation. 2002;106:2486-2490
doi: 10.1161/01.CIR.0000036599.31371.8F
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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