Obesity-Induced Hypertension Develops in Young Rats Independently of the Renin-Angiotensin-Aldosterone System

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A correlation exists between obesity and hypertension. In the currently available models of diet-induced obesity, the treatment of rats with a high fat (HF) diet does not begin until adulthood. Our aim was to develop and characterize a model of pre-pubescent obesity–induced hypertension. Male Sprague-Dawley rats were fed a HF diet (35% fat) for 10 weeks, beginning at age 3 weeks. Blood pressure was measured by tail-cuff, and a terminal blood sample was obtained to measure fasting blood glucose, insulin, plasma renin, aldosterone, thiobarbituric acid reactive substances (TBARS), and free 8-isoprostanes levels. The vascular reactivity in the aorta was assessed using a myograph. Blood pressure was increased in rats fed the HF diet (HF, 161 ± 2 mm Hg vs. control, 137 ± 2 mm Hg, $P < 0.05$). Blood glucose (HF, 155 ± 4 mg/dL vs. control, 123 ± 5 mg/dL, $P < 0.05$), insulin (HF, 232 ± 63 p*M* vs. control, 60 ± 11 p*M*, $P < 0.05$), TBARS (expressed as n*M* of malondialdehyde [MDA]/ml [HF, 1.8 ± 0.37 n*M* MDA/ml vs. control 1.05 ± 0.09 n*M* MDA/ml, $P < 0.05$]), and free 8-isoprostanes (HF, 229 ± 68 pg/ml vs. control, 112 ± 9 pg/ml, $P < 0.05$) levels were elevated in the HF diet group. Interestingly, plasma renin and aldosterone levels were not different between the groups. The maximum vasoconstriction to phenylephrine (10^{-4} *M*) was increased in the HF diet group (HF, 26.1 ± 1.5 mN vs. control 22.3 ± 1.2 mN, $P < 0.05$). In conclusion, pre-pubescent rats become hypertensive and have increased oxidative stress and enhanced vasoconstriction when fed a HF diet. Surprisingly, this occurs without the increase in renin or aldosterone levels seen in the adult models of diet-induced obesity. Exp Biol Med 231:282–287, 2006

Key words: obesity; hypertension; vasoconstriction; phenylephrine; adrenal steroids; reactive oxygen species

Introduction

In the United States 61% of the adult population is considered overweight or obese, and each year 400,000 obesity-associated deaths occur. It is estimated that $122.9 billion is spent annually on obesity-related health issues, such as heart disease, type II diabetes, stroke, and hypertension (1). It is clear that there is a strong correlation between the incidence of hypertension and obesity (2), more specifically visceral and abdominal obesity (3–5). Data from the Framingham Heart Study show that approximately 78% of essential hypertension in men and approximately 65% of essential hypertension in women can be directly attributed to obesity (6). Dobrian *et al.* showed that a rat model of diet-induced obesity, used to study hypertension, closely mimics the cardio-renal changes found in obese humans. In this model, there was a 30% increase in aortic wall thickness in obese rats compared with controls, the renin-angiotensin system was activated, and there was evidence of mild glomerulosclerosis (7). These physiological and structural changes are also seen in human hypertension (8–10). However, in this rodent model of diet-induced obesity, the rats were adult when they began the treatment with a high-fat (HF) diet, making this model unsuitable for studying pre-pubescent or juvenile obesity. The incidence of obesity in children is increasing at an alarming rate, with a prevalence of 21.5% in black, 21.8% in Hispanic, and 12.3% in non-Hispanic white children (11). Our aim was to develop and characterize a model of diet-induced obesity and to assess the effects of childhood obesity on the adult cardiovascular system. In other models of hypertension there are changes in vascular reactivity (12–19). Therefore, we wanted to test the novel hypothesis that feeding pre-pubescent rats a HF diet would result in hypertension and altered vascular reactivity.

Materials and Methods

Three-week-old, male Sprague-Dawley rats (35–49 g body wt; Harlan, Indianapolis, IN) were used. All procedures were approved by the Medical College of Georgia's Animal Use for Research and Education Committee, and all protocols followed the American Physiological Society's guidelines on animal use. Rats were randomly separated into

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two groups; one group was fed regular rat chow (4.4% fat, 0.39% sodium, and 1.0% potassium; Harlan), and the second was fed a HF diet (35.5% fat, 0.4% sodium, and 0.6% potassium; Bioserve, Frenchtown, NJ) for 10 weeks. Rats were maintained on a 12:12-hr light:dark cycle and had access to chow and water *ad libitum*. Systolic blood pressure was measured by tail-cuff (pneumatic transducer). Thirty-eight HF and 21 control rats were treated, and a subset of these rats was used for biochemical and vascular reactivity analysis.

Rats were fasted overnight and then euthanized with sodium pentobarbital (50 mg/kg, ip). Blood was collected by cardiac puncture to measure fasting blood glucose levels (Abbot Laboratories, Bedford, MA). Plasma renin activity (Diasorin Inc., Stillwater, MN), aldosterone, free 8-iso-prostanes (Cayman Chemicals, Ann Arbor MI), and thiobarbituric acid reactive substances (TBARS) (ZeptoMetrix Corp., Buffalo, NY) were also studied, and all assays were performed following the manufacturer's protocols. Urinary glucose was measured using a urine chemistry analyzer (Clinitek 50; Bayer, Pittsburgh, PA). The wet weights of the visceral fat (total retroperitoneal, epididymal, and mesenteric fat) and heart were also measured. Subcutaneous fat was not measured.

Vascular Reactivity Studies. The aorta was excised, placed in cold physiological salt solution (PSS), and cleaned of connective tissue (PSS; composition in m*M*: NaCl 130.0, KCl 4.7, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 14.9, dextrose 5.5, EDTA 0.026, $CaCl_2$ 1.6). Three- to 4-mm rings were cut and mounted in a myograph (model 610M; Danish Myo Technology, Copenhagen, Denmark) to study isometric force generation; to obtain endothelium-denuded rings, the endothelium was removed by gently rubbing the lumen surface. The myograph chamber was filled with 37°C PSS and bubbled with 95% O_2 and 5% CO_2, and a passive tension of 39 mN was placed on the rings. Vessels were allowed to equilibrate for 45 mins in the presence of indomethacin (10^{-5} m*M*) before being challenged with phenylephrine (PE, 10^{-7} m*M*), to ensure viability, and acetylcholine (ACH, 10^{-5} m*M*), to determine the status of the endothelium. Cumulative dose-response curves to PE (0.1 n*M*–0.1 m*M*) were generated. Vessels were then preconstricted with an EC70 dose of PE (10^{-7} m*M*), and relaxation was assessed by performing a dose-response curve to ACH (0.1 n*M*–0.1 m*M*) in intact vessels and to sodium nitroprusside in denuded vessels (0.1 n*M*–0.1 m*M*).

Western Blot Analysis. Protein expression of cyclooxygenase-2 (COX-2) was determined by Western blot analysis. The aorta was removed, snap frozen, and stored at −80°C until use. Protein was extracted from the aorta by homogenization in modified radioimmunoprecipitation buffer and the homogenate was centrifuged. Samples containing 100 μg of total protein, combined with loading buffer (Bio-Rad, Hercules, CA) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to polyvinylidene difluoride membrane. Nonspecific binding sites on the membrane were blocked with 0.1% casein in phosphate-buffered saline for 2 hrs at room temperature. The membrane was then incubated overnight at 4°C with a polyclonal anti-rabbit COX-2 antibody (1:1000; Cayman Chemicals, Ann Arbor, MI). The membrane was then incubated with an Alexa Fluor 680 goat anti-rabbit secondary antibody (1:5000; Molecular Probes, Carlsbad, CA) for 1 hr. Bands were visualized using an infrared imaging system (LI-COR, Inc., Lincoln, NE), and the fluorescence intensity was quantified. The membrane was then stripped using Re-blot plus (Chemicon International, Inc., Temecula, CA) and then reprobed using a primary monoclonal anti-β tubulin antibody (1:1000; Sigma Chemical Company, St. Louis, MO). The membrane was then incubated in an IRDye 800 goat anti-mouse secondary antibody (1:5000; Rockland, Gilbertsville, PA). The results obtained for COX-2 expression were normalized using the results from β-tubulin expression.

Reagents. Unless otherwise stated, all chemicals were purchased from Sigma. A stock solution of indomethacin was dissolved in ethanol (<0.1%, final concentration in organ chamber). All other chemicals were dissolved in distilled water.

Statistical Analyses. Results are presented as mean ± SEM. An analysis of variance was used for multiple comparisons followed by a Bonferroni *post hoc* test when necessary. Student's *t* test was used when analyzing two values when appropriate. The significance level was set at $P < 0.05$.

Results

Physiological Variables. At the end of the 10-week treatment, rats fed the HF diet had an approximately 17% increase in blood pressure compared with control rats. Fasting blood glucose levels were elevated in the rats fed the HF diet compared with control rats. Fasting insulin levels were also higher from the rats fed the HF diet, suggesting the rats were insulin resistant (Table 1). However, there was no trace of urinary glucose in either group, indicating that the rats fed the HF diet were not overtly diabetic. The overall body weight was 16% greater in the HF rats, and there was more than two-fold increase in total visceral fat. The visceral fat to body weight ratio and heart weight in the HF diet group were also significantly increased (Table 2). Neither the plasma renin nor the aldostreone levels were increased in the rats fed the HF diet compared with the control rats. TBARS and free 8-isoprostanes in the plasma from the rats fed the HF diet were elevated, indicating increased oxidative stress (Table 1).

Effect of a High-Fat Diet on Vascular Reactivity in the Aorta. To examine vasoconstriction in the aorta, the responsiveness to PE was assessed. Constriction to PE was augmented in aortas from rats fed a HF diet, and this enhanced constriction became apparent at the concentration of 1 μ*M* of PE. Maximum constriction was significantly increased in the HF group ($n = 7$, 26.1 ± 1.5 mN) compared

Table 1. Systolic Blood Pressure, Urine, and Blood Chemistry in Rats Fed a High Fat (HF) Diet or Regular Rat Chow for 10 Weeks. The Number of Animals Used for Each Parameter Is Indicated in Parentheses. ND Indicates None Detected.

	Control	HF
Blood pressure (mm Hg)	137 ± 2 ($n = 21$)	161 ± 2 ($n = 35$)*
Blood glucose (mg/dl)	123 ± 5 ($n = 12$)	155 ± 4 ($n = 29$)*
Urinary glucose	ND	ND
Plasma insulin (p*M*)	60 ± 11 ($n = 9$)	232 ± 63 ($n = 8$)*
Plasma adlosterone (pg/dl)	8.97 ± 0.72 ($n = 10$)	8.66 ± 0.66 ($n = 10$)
Plasma renin (ng Angl/ml/hr)	18.75 ± 0.83 ($n = 12$)	18.12 ± 0.39 ($n = 10$)
TBARS (nmol MDA/ml)	1.05 ± 0.48 ($n = 7$)	1.80 ± 0.37 ($n = 8$)*
Plasma 8-isoprostanes (pg/ml)	112 ± 9 ($n = 9$)	229 ± 68 ($n = 8$)*

*$P < 0.05$ compared with controls (Student's *t* test).

with the control group ($n = 8$, 22.3 ± 1.2 mN; Fig. 1). However, there was not a shift in the EC50 between groups.

To examine vasorelaxation we used ACH to test endothelium-dependent relaxation and sodium nitroprusside (SNP) to test endothelium-independent relaxation in the aorta. Relaxation to ACH was conducted in endothelium-intact vessels, whereas SNP-treated vessels were denuded. Vessels were preconstricted with PE (10^{-7} *M*). There was not a significant difference in endothelium-dependent or -independent relaxation as evaluated by ACH ($n = 7$ in each group; Fig. 2A) and SNP ($n = 7$ in each group; Fig. 2B).

Western blot analysis was used to determine the expression of the inducible cyclooxygenase COX-2. Protein was extracted from the aorta from the rats fed the HF diet ($n = 7$) and control rats ($n = 8$). There was not an increase of COX-2 in the aorta from the rats fed the HF diet compared with the control rats (HF, 3.05 ± 1.24 vs. control, 1.97 ± 0.99, arbitrary units, corrected for β-tubulin expression, $P > 0.05$).

Discussion

There are three major findings in the current study. First, beginning a HF diet before puberty results in an increase in blood pressure despite there being a smaller increase in body weight than what is seen in adult rats fed a HF diet. Second, plasma renin and aldosterone levels were normal in the rats fed the HF diet. Finally, the vasoconstrictor response to PE was enhanced in the aortas of rats fed a HF diet.

This is not the first study to use a HF diet to induce visceral obesity and hypertension in rats; studies from Dobrian *et al.* have also shown that feeding rats a HF diet increases blood pressure (7). The major difference between the studies reported here and those of Dobrian *et al.* is the age at which the rats began the HF diet. Although no age was reported for the rats in the studies from Dobrian *et al.*, the rats had a body weight of 300–350 g at the beginning of the study, suggesting these animals were approximately 16–18 weeks old. In our studies, rats began eating the HF diet at age 3 weeks. Despite having a smaller reported increase in body weight (16% vs. 25%), the young animals had a similar increase in blood pressure compared with the older animals.

Interestingly, another difference between the studies using young and adult rats is the rate at which hypertension occurs in the rats fed a HF diet. In the Dobrian *et al.* experiment, 50% of the rats placed on the HF diet became obese and the others were deemed obesity resistant (7). The authors report that the obesity-resistant rats did not become hypertensive. In our young rats fed a HF diet, only three of the 38 rats studied did not develop hypertension; these rats were removed from the study. In these studies systolic blood pressure was measured, and any reading higher than 140 mm Hg was considered hypertensive. The young rats became hypertensive at age 7 weeks. This difference in the rates in which hypertension develops suggests that a physiological change occurs before the rats reach adulthood, which either predisposes or protects them from obesity. Zhou *et al.* have shown that young female rats fed a HF diet do not develop hypertension; however, when treated with 5α-dihydrotestosterone and the HF diet, the mean arterial

Table 2. Body, Total Visceral Fat, and Heart Weights in Rats Fed a High-Fat (HF) Diet or Regular Rat Chow for 10 Weeks. The Number of Animals Used for Each Parameter Is Indicated in Parentheses.

	Control	HF
Body weight (g)	353 ± 4 ($n = 23$)	408 ± 5 ($n = 29$)*
Visceral fat weight (g)	6.9 ± 0.2 ($n = 19$)	15.6 ± 0.8 ($n = 25$)*
Fat/body weight	2.0 ± 0.07 ($n = 19$)	3.8 ± 0.18 ($n = 25$)*
Heart weight (g)	1.2 ± 0.03 ($n = 19$)	1.4 ± 0.02 ($n = 26$)*
Heart/body weight*100	0.35 ± 0.01 ($n = 19$)	0.33 ± 0.01 ($n = 26$)*

*$P < 0.05$ compared to control (Student's *t* test).

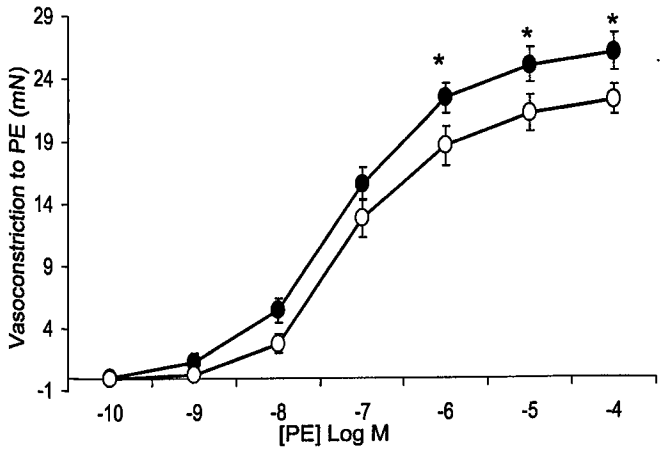

Figure 1. Dose response curves in aortic rings from rats fed a high-fat ($n = 7$, HF) diet or control chow ($n = 8$) to phenylephrine (PE, 0.1 n*M* – 0.1m*M*). Closed circles represent the data from rats fed the HF diet and open circles, control rats. * $P < 0.05$ compared with controls. (ANOVA, followed by a Bonferroni multiple comparison test).

pressure is increased (20). Therefore, it is possible that androgens are responsible for this effect, but further investigations are needed. Regardless, it is clear from the studies reported here that beginning a HF diet at an early age may result in an increased risk of an adverse cardiovascular outcome. It is not clear if the same age dependence exists in humans. If it does, however, the current epidemic of childhood obesity will result in a dramatic increase in cardiovascular disease as these children become adults.

One of the other interesting differences between adult and young rats is the response of the renin-angiotensin-aldosterone system (RAAS) in the rats fed the HF diet. In the studies reported here, the renin and aldosteone levels were similar in the rats fed the HF diet and control rats, suggesting that the RAAS is not activated in our model of diet-induced hypertension. This is a contradiction to many previous studies of obesity-induced hypertension. Studies of canine obesity-induced hypertension have shown that aldosterone antagonism prevents the hypertension induced by a HF diet, suggesting that aldosterone plays an important role in the pathogenesis of obesity-induced hypertension. It is possible that an increase in sympathetic drive accounts for the activiation of the RAAS in these studies (21). The absence of elevated plasma renin and aldosterone suggests that the sympathetic nervous system may not be activated in our young rats. Others have shown that obese humans have elevated aldosterone levels irrespective of their blood pressure (22–25). Interestingly, some recent studies have suggested that adipocytes release an as of yet unidentified factor that increases aldosterone production (26). That given, one would expect that young rats that have visceral obesity would also have elevated plasma aldosterone levels. This, as we mentioned previously, was not the case. One possibility is that the adipocytes from young animals are physiologically different in that they do not release factors to stimulate aldosterone secretion.

Previous studies of various forms of hypertension have shown that vascular reactivity is altered. Many studies have shown that the response to vasoconstrictor agonists is increased in hypertensive models (12–19). In particular, there are alterations to α-adrenergic responses that were determined with PE (27–31). We also observed an increase in the response to PE in the aorta. Although we observed an increase in the isometric force generated in response to PE in rats fed a HF diet, we did not observe a change in the EC50 for PE. This suggests that there is not an alteration in the sensitivity of the α-adrenergic receptors to PE but that there may be an increase in the smooth muscle mass generating the contractile force. This fits with previous studies that have shown a 30% increase in vessel wall mass

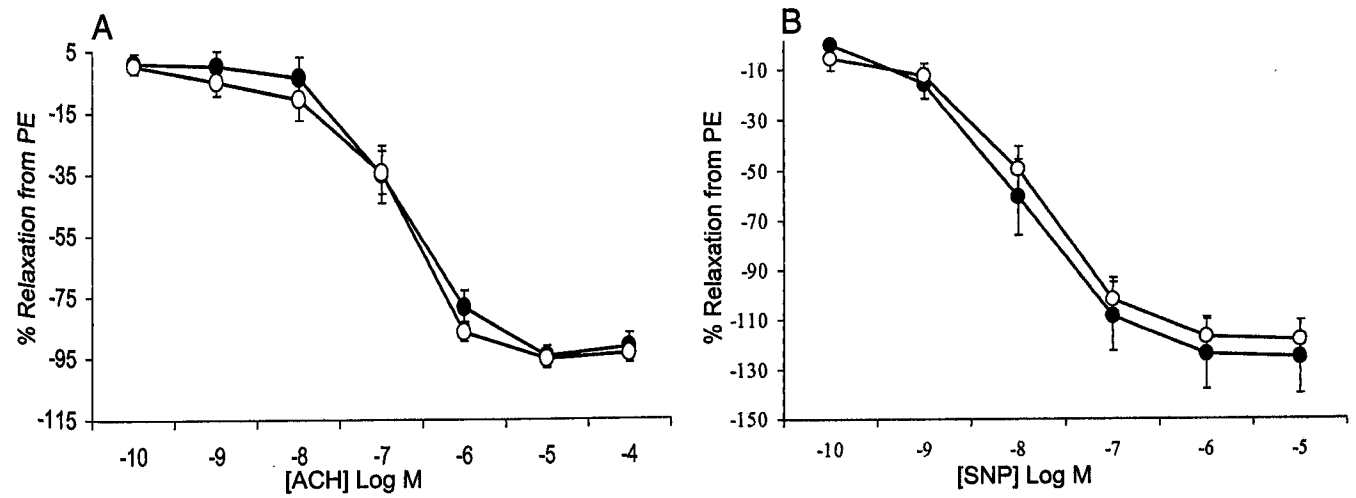

Figure 2. (A) Dose response curves to acetylcholine (ACH, 0.1 n*M* – 0.1m*M*) after preconstriction to the EC70 of phenylephrine (PE). The force generated in the aorta in response to an EC70 dose of PE was 14.01 ± 1.18 mN for the rats fed the HF diet and 12.25 ± 1.20 mN for the controls ($n = 7$ in each group). (B) Dose response curves to sodium nitroprusside (SNP, 0.1 n*M* – 0.1m*M*) after preconstriction to PE. The force generated in the aorta in response to an EC70 dose of PE was 12.62 ± 1.64 mN for the rats fed the HF diet and 12.28 ± 1.88 mN for the controls. Closed circles represent the data from the rats fed the HF diet and open circles, control rats ($n = 7$ in each group).

in rats fed a HF diet (7). If this were the case, one would expect an increase in contraction and not an increase in sensitivity as seen here. One of the limitations of the current study is that only one vasoconstrictor agonist was tested in the aortas. We have tested the effects of serotonin and clonidine in mesenteric arteries, and no difference in vascular reactivity was observed (data not shown).

ACH stimulates endothelial nitric oxide synthase (eNOS) to produce nitric oxide and increase cGMP production, resulting in smooth muscle relaxation. We did not find any differences in ACH-induced endothelium-dependent relaxation in the aorta in this model despite the development of hypertension. This result was unexpected because there was an increase in plasma TBARS and free 8-isoprostanes, indicators of oxidative stress thought to lead to endothelial dysfunction. Other investigators have also observed increases in markers of oxidative stress in models of obesity (7, 32–34). Furthermore, in animal models of obesity, increases in eNOS mRNA expression have been observed in the thoracic aorta and kidney cortex and mudella (7). In another study, investigators found that renal eNOS was increased in female rats after 6 months on a HF diet, only to decline after the rats had been on the diet for 2 years (35). We did not measure eNOS expression or activity. However, because no difference in ACH was observed, this suggests that eNOS may be unchanged or upregulated as a compensatory mechanism in the rats fed the HF diet. It is also possible that although oxidative stress was increased in the HF group, 10 weeks may not have been enough time to cause endothelial damage. In some animal models of hypertension, there is also an increase in protein expression of COX-2 (36–38). Interestingly, COX-2 protein expression in the aorta was not increased in the rats fed the HF diet.

There was no difference in the relaxation between vessels from the rats fed a HF diet and the control group to SNP, a NO donor. By denuding the vessels, the endothelium layer responsible for producing endogenous NO is destroyed. Exogenous NO can then be used to assess the sensitivity of downstream mediators of NO-mediated relaxation. The absence of an effect of the HF diet on SNP-mediated vasodilation suggests that the downstream mediators of NO remain unaltered.

These studies are particularly timely given the increasing population suffering from juvenile obesity. Our model shows that there is an increase in reactivity to PE, blood pressure, blood glucose levels, insulin, visceral fat, heart size, and oxidative stress at an early age. More important, the young rats appear to develop hypertension without the activation of the RAAS, suggesting that the pathology of the disease is different in young versus old rats. This may lead to different therapeutic treatments for younger individuals with obesity-induced hypertension compared with adults with obesity-induced hypertension.

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