Increased Muscle Mass Protects Against Hypertension and Renal Injury in Obesity

Joshua T. Butcher, PhD; James D. Mintz, MBA; Sebastian Larion, MD; Shuiqing Qiu, PhD; Ling Ruan, MD; David J. Fulton, PhD; David W. Stepp, PhD

Background—Obesity compromises cardiometabolic function and is associated with hypertension and chronic kidney disease. Exercise ameliorates these conditions, even without weight loss. Although the mechanisms of exercise's benefits remain unclear, augmented lean body mass is a suspected mechanism. Myostatin is a potent negative regulator of skeletal muscle mass that is upregulated in obesity and downregulated with exercise. The current study tested the hypothesis that deletion of myostatin would increase muscle mass and reduce blood pressure and kidney injury in obesity.

Methods and Results—Myostatin knockout mice were crossed to *db/db* mice, and metabolic and cardiovascular functions were examined. Deletion of myostatin increased skeletal muscle mass by ≈50% to 60% without concomitant weight loss or reduction in fat mass. Increased blood pressure in obesity was prevented by the deletion of myostatin, but did not confer additional benefit against salt loading. Kidney injury was evident because of increased albuminuria, which was abolished in obese mice lacking myostatin. Glycosuria, total urine volume, and whole kidney NOX-4 levels were increased in obesity and prevented by myostatin deletion, arguing that increased muscle mass provides a multipronged defense against renal dysfunction in obese mice.

Conclusions—These experimental observations suggest that loss of muscle mass is a novel risk factor in obesity-derived cardiovascular dysfunction. Interventions that increase muscle mass, either through exercise or pharmacologically, may help limit cardiovascular disease in obese individuals. (***J Am Heart Assoc***. 2018;7:e009358. DOI: 10.1161/JAHA.118.009358.)

Key Words: hyperglycemia • hypertension • myostatin • nicotinamide-adenine dinucleotide phosphate, reduced form, oxidase 4 • skeletal muscle

T he obesity epidemic in the United States, defined as the prevalence of overweight/obese adults with a body mass index ≥25 kg/m^2, now approaches ≈70% of the population.[1,2] Obesity is a major risk factor for high blood pressure, which has been identified as a leading cause for cardiovascular disease deaths in the United States, and thus the target of new guidelines recently released by the American College of Cardiology/American Heart Association.[3] Although numerous pharmacotherapies exist for hypertension, cures remain elusive and the aspects of obesity that drive hypertension remain undefined. A key intervention that improves blood pressure regulation in obese individuals is exercise, but the mechanisms by which exercise has its beneficial effects are unclear.[4,5]

One potential mechanism by which exercise benefits hypertensive patients is through an increase in the quantity or quality of skeletal muscle. Skeletal muscle mass is regulated by myostatin, a member of the transforming growth factor-β superfamily and a potent negative regulator of muscle growth.[6–8] Myostatin is upregulated in obesity and downregulated by exercise.[9–12] Targeted inhibition of myostatin, genetically or pharmaceutically, has had promising results in improving cardiometabolic health (namely, glucose tolerance) and preventing/restoring muscle mass in rodents and humans.[8,13–18]

In the current study, we test the hypothesis that restoration of muscle mass in obese mice via deletion of myostatin will improve regulation of arterial pressure. Mice harboring a genetic deletion of myostatin were bred to leptin receptor mutant *db/+* mice to produce a strain of lean and obese hypermuscular animals, compared with their respective controls. Blood pressure was measured by long-term

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Clinical Perspective

What Is New?

- Novel findings are that increases in muscle mass prevent obesity-associated hypertension and that renal injury is ameliorated, as indicated by reduced albuminuria.
- Further studies identified nicotinamide-adenine dinucleotide phosphate, reduced form, oxidase 4 as a potential link between muscle mass and renal function and that increasing muscle mass alleviates the burden of glycemia/glycosuria and excess fluid load (diabetic polyuria and polydipsia) on the kidney.

What Are the Clinical Implications?

- The significance of these findings is that they identify a new factor, loss of muscle mass, as a contributing factor to obesity-related hypertension.
- This observation opens the door to new avenues of treating high blood pressure and may explain why certain populations without muscle loss (young adults) are protected against hypertension compared with those with muscle loss (the aged).

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telemetry, and renal function was assessed in metabolic chambers. Extensive hormonal phenotyping was conducted, and lipemic and glycemic status was determined. The role of salt, long indicated as a key player in hypertension, was examined via dietary manipulation of sodium. Obesity increases oxidant stress; thus, renal expression of oxidant genes was measured by reverse transcription–polymerase chain reaction. These studies provide the first assessment of muscle mass as a predictor of blood pressure control in obese animals.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. All experiments were approved by the Augusta University (Augusta, GA) Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. Male adult mice (19.1 ± 0.8 weeks old) were used for the duration of this experiment. The *db/db* mouse was selected because it produces a reliable hypertensive response and to avoid the confounding effects of weight loss associated with the impact of myostatin in diet-induced obesity models.[19–24] To generate animals for this study, myostatin knockout mice were crossed onto the *db/db* background, as previously described.[25] Nomenclature of the mice reflects whether each gene is heterozygous (H) or knocked out/mutated (K) for the *db/db*

leptin receptor mutation (first letter) or the myostatin gene (second letter). An $H_{db}H_{myo}$ mouse is heterozygous for both the *db/db* mutation and the myostatin deletion, serving as the lean control. A $K_{db}K_{myo}$ mouse is homozygous for both the *db/db* mutation and a myostatin knockout, representing an obese muscular mouse.

Hemodynamics were obtained using in vivo radiotelemetry in conscious freely moving mice. Transmitters (PA-C10) were obtained from Data Sciences International and implanted for measurement of blood pressure, as described previously.[26] Mice were anesthetized with isoflurane, a transmitter cannula was threaded down the left carotid, and its battery pack was tunneled into the animal's right back. Animals recovered for 7 days, after which baseline recordings for mean arterial pressure, systolic and diastolic pressure, heart rate, and activity were continuously collected (10 s/min) for 7 days.

Body and tissue composition were measured using a Bruker minispec LF90 II TD-NMR. Oxygen consumption and carbon dioxide production were obtained using a Columbus Instrument Comprehensive Lab Animal Monitoring System (CLAMS). Animals were monitored over 4 days in the system, and energy expenditure, activity, and heat were recorded. Respiratory exchange ratio was calculated using the equation carbon dioxide production/oxygen consumption. Baseline food and water consumption, along with urine production, was obtained using metabolic cages. The animals were acclimated to metabolic cages for 24 hours, and then daily food and water consumption, and urine production, was obtained for 2 days. Urine was collected daily, centrifuged, and stored at $-80°C$. Urine electrolyte analysis was conducted using an Easylyte Plus Na/K/Cl/Li Analyzer from Medica.

Two groups of mice were used for salt sensitivity studies, one group for kidney function and one group for blood pressure. All diets were purchased from Envigo Teklab Custom Diets. After baseline measurements with normal chow (0.4% NaCl) were obtained, each group was exposed to 3 days of high-salt diet (4% NaCl), allowed to recover back to baseline for 5 days, and then placed on a low-salt diet (0.1% NaCl).

Whole blood was collected from fasted animals in heparinized tubes and centrifuged at 4°C for 10 minutes at 8K RPM, and the plasma was stored at $-80°C$ for analysis. Plasma and urine were analyzed using the following commercially available kits: cholesterol (Cholesterol, Total), triglyceride (L-Type Triglyceride M), microalbumin, and nonesterified fatty acid kits were obtained from Wako Diagnostics; insulin (Mouse Insulin ELISA), leptin (Mouse/Rat Leptin ELISA), insulin-like growth factor 1, tumor necrosis factor-α, interleukin-10, adiponectin (Mouse) total, and high-molecular-weight ELISA were obtained from ALPCO; glucose (ALPHATRAK 2) was obtained from Andwin Scientific; vasopressin was obtained with Cusabio's mouse ADH/VP/AVP

ELISA; and hemoglobin A1c was obtained with a PD Diagnostic A1C multitest AK system. Urinary creatinine and glucose were assessed using kits from Caymen Chemical. Estimated glomerular filtration rate (GFR) was calculated using the following equation: $GFR = U(Cr) \times \dfrac{Volume}{Time} / P(Cr)$, where U (Cr) is urine creatinine, and P(Cr) is plasma creatinine. The UMass University of Massachusetts Mouse Metabolic Phenotyping Cente (MMPC) and Vanderbilt Hormone Assay and Analytical Services Core (National Institutes of Health grants DK059637 and DK020593) performed the blood urea nitrogen, creatinine, uric acid and aldosterone, amino acid, and muscle lipid analyses. Urinary hydrogen peroxide was assessed using the Invitrogen Amplex Red Hydrogen Peroxide Assay Kit and expressed as relative fluorescent units (arbitrary unit).

Gene expression for the nicotinamide-adenine dinucleotide phosphate, reduced form, oxidase (NOX) isoforms was conducted on total kidney tissue homogenates, as described previously.[27] Briefly, total RNA was extracted using the RNeasy mini kit from Qiagen (no. 74104). Synthesis of cDNA occurred using SuperScript III Reverse Transcriptase from Invitrogen (no. 18080-093). Primers used for analysis were designed with BLAST. A Bio-Rad CFX Connect Realtime PCR detector was used for quantitative reverse transcription–polymerase chain reaction in combination with SsoAdvanced Universal SYBR Green (Bio-Rad no. 172-5271). Expression of target genes was normalized to an internal reference gene (GAPDH) and the control group (HH) and reported as fold changes using the $2^{-\Delta\Delta Ct}$ method.

All data are expressed as mean±SEM. Results were analyzed using Graphpad Prism 7.03, and significance was determined at $P<0.05$ using a 1-way ANOVA with Tukey's multiple comparison test or unpaired Student t test, where appropriate. Significance is noted in the legends and defined as follows: $*P \leq 0.05$ versus $H_{db}H_{myo}$, $^{\#}P \leq 0.05$ versus $K_{db}H_{myo}$, and $^{@}P \leq 0.05$ versus $H_{db}K_{myo}$.

Results

Myostatin Deletion Increases Muscle Mass in Obesity Independent of Changes in Body Weight or Fat Content

Anatomic characteristics of all 4 groups of mice are described in Figure 1 and Table 1. Consistent with previously published
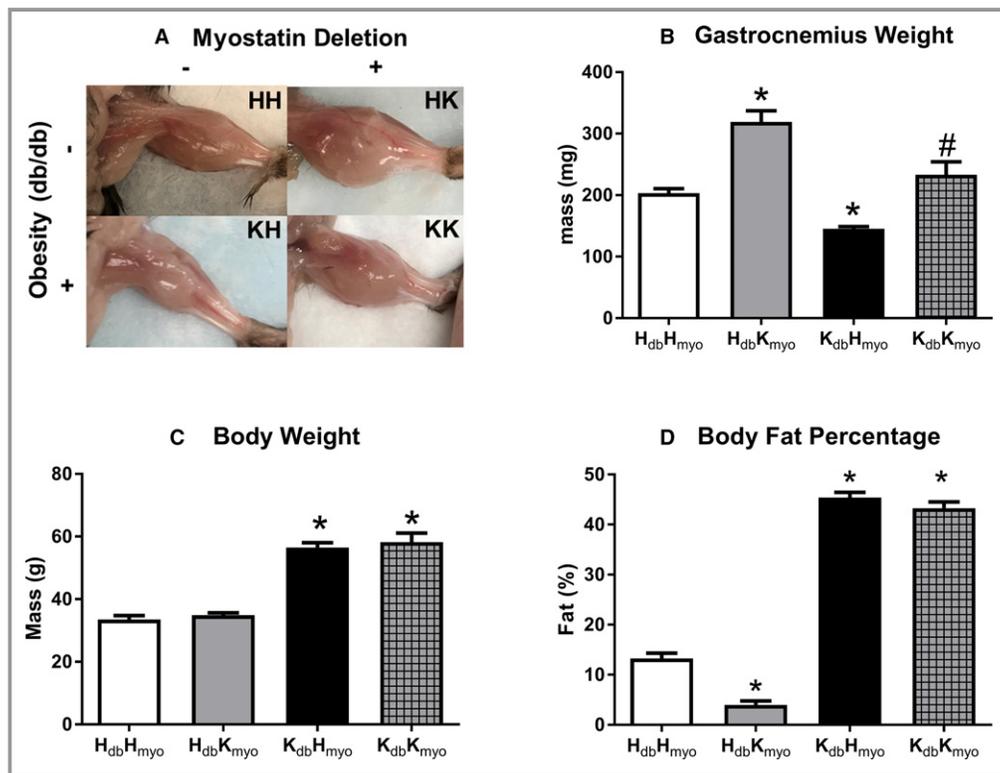
Figure 1. Myostatin deletion increases muscle mass independent of mass and adiposity. A, A representative image of the gastrocnemius muscle for all groups. B, Myostatin deletion significantly augments muscle mass in lean and obese groups. Body weight (C) and whole body adiposity (D) are unchanged between obese mice. N≥10 for each group. H indicates heterozygous; H_{db}, H for the *db/db* leptin receptor mutation; H_{myo}, H for the myostatin gene; K, knocked out/mutated; K_{db}, K for the *db/db* leptin receptor mutation; and K_{myo}, K for the myostatin gene. $*P \leq 0.05$ vs HH; $^{\#}P \leq 0.05$ vs KH.

data, myostatin deletion significantly increases muscle mass in both the lean and obese mice compared with their respective controls.[25] Although whole body fat decreases in $H_{db}K_{myo}$ mice compared with the $H_{db}H_{myo}$ mice, the *db/db*

mice have comparable whole body fat content with or without myostatin expression, as assessed either as total body fat or percentage fat. Using the gastrocnemius muscle as representative, we observed increased muscle mass in $H_{db}K_{myo}$

Table 1. Anatomic Profile

Variable	$H_{db}H_{myo}$	$H_{db}K_{myo}$	$K_{db}H_{myo}$	$K_{db}K_{myo}$
Snout to anus length, cm	10.4±0.4	9.8±0.01	10.2±01	10.2±0.1
Tibia length, mm	19.7±0.2	19.1±0.1	19.4±0.1	19.1±0.2
Heart, mg	155±4.9	155±4.0	166±3.6	168±5.5
Kidney, mg	224±13	217±11	261±12*	224±15[†]
Whole body fat, g	5.5±0.6	1.5±0.3*	26.6±1.2*	26.3±1.6*
Muscle characteristics				
Gastrocnemius fat, %	2.3±0.4	1.0±0.6	14.3±1.2*	10.0±1.4*[†]
Free fatty acids, μg/mg	0.57±0.09	0.42±0.08[†]	0.79±0.12	0.49±0.04[†]
Triglycerides, μg/mg	39.8±18.2	29.2±11.8[†]	167.4±38.8*	25.7±4.3[†]
Cholesterol, μg/mg	0.17±0.03	0.10±0.008	0.18±0.04	0.11±0.009
Whole body metabolism				
VO_2, mg/kg per hour	3221±99	3197±164	2184±138*	2067±198*
VCO_2, mg/kg per hour	2904±135	2961±172	1947±125*	1914±189*
Heat, kcal/h	0.56±0.01	0.55±0.02	0.59±0.03	0.51±0.03
Respiratory exchange ratio	0.90±0.03	0.90±0.01	0.89±0.1	0.91±0.1
Voluntary activity (wheel count)	1672±301	1396±398	189±57*	133±58*
Average daily activity, AU	9.5±1.7	9.8±1.7	1.8±0.7*	1.6±0.4*
Plasma indexes of adiposity and insulin resistance				
Hemoglobin A1c, %	4.6±0.1	4.5±0.1	7.8±0.4*	6.1±0.3*[†]
Fasting plasma glucose, mg/dL	216±14	193±10	309±23*	225±23[†]
Leptin, pg/mL	591±39	244±21*	1970±69*	1825±69*
Insulin, ng/mL	0.50±0.05	0.39±0.07	3.08±0.53*	2.63±0.33*
IGF-1, ng/mL	33.6±4.9	35.8±10.1	22.8±3.2	39.4±5.5
Cholesterol, mg/dL	60.1±2.5	53.5±3.6	129.7±10.9*	106.9±14.3*
Triglycerides, mg/dL	31.3±5.6	40.7±6.8	105.8±10.8*	87.9±33.3
NEFA, mEq/mL	0.7±0.1	0.6±0.2	0.9±0.1	0.9±0.1
Indexes of oxidative stress and inflammation				
TNF-α, pg/mL	40.5±3.9	19.3±4.1	85.2±5.5*	77.0±9.9*
Interleukin-10, pg/mL	150.5±14	127.7±12	92.0±15	87.5±21
Uric acid, μmol/L	36.3±1.9	37.3±5.6	45.5±6.8	63.7±5.4*[‡]
Glycosuria, mg/dL per day	0.04±0.01	0.08±0.03	4.7±2.4*	1.2±0.5[†]
GFR, mL/min	0.87±0.10	0.73±0.14	0.97±0.10	0.69±0.14
Urinary H_2O_2, AU/μL	2431±957	3691±1415	10 004±2593*	3651±978
Plasma hormones				
Blood urea nitrogen, mg/dL	19.3±2.3	21.3±2.6	17.0±2.1	22.8±0.6
Plasma creatinine, mg/dL	0.08±0.004	0.13±0.016*[†]	0.058±0.005	0.074±0.006
Urinary creatinine, mg/d	0.76±0.10	1.3±0.09	0.90±0.08	0.84±0.26

Continued

Table 1. Continued

Variable	$H_{db}H_{myo}$	$H_{db}K_{myo}$	$K_{db}H_{myo}$	$K_{db}K_{myo}$
Microalbuminuria, μg/d	231±64	149±26	892±180*‡	200±49†
Total plasma adiponectin, ng/mL	157±19	109±25	111±36	53±8*
HMW plasma adiponectin, ng/mL	24±4	21±5	24±8	5±0.4
Aldosterone, pg/mL	268.5±42	238.1±31	342.3±51	270.0±40
Vasopressin, pg/mL	27.1±4.5	23.3±2.5	36.1±2.4‡	29.6±3.5

Data are given as mean±SEM. N=6 to 12 for each group. GFR indicates estimated glomerular filtration rate; H_{db}, heterozygous for the *db/db* leptin receptor mutation; HMW, high molecular weight; H_{myo}, heterozygous for the myostatin gene; IGF-1, insulin-like growth factor 1; K_{db}, knocked out/mutated for the *db/db* leptin receptor mutation; K_{myo}, knocked out/mutated for the myostatin gene; NEFA, nonesterified fatty acid; TNF-α, tumor necrosis factor-α; VCO_2, carbon dioxide production; VO_2, oxygen consumption.
*$P \leq 0.05$ vs HH.
†$P \leq 0.05$ vs KH.
‡$P \leq 0.05$ vs HK.

mice and reduced muscle mass in obese $K_{db}H_{myo}$ mice. In obese $K_{db}K_{myo}$ mice, muscle was increased to the size of muscle in control $H_{db}H_{myo}$ mice. The muscle of $K_{db}H_{myo}$ mice also displayed increased fat content via nuclear magnetic resonance and increased free fatty acid and triglyceride content, but not cholesterol accumulation. In parallel with

Table 2. Plasma Amino Acid Profile

Variable	$H_{db}H_{myo}$	$H_{db}K_{myo}$	$K_{db}H_{myo}$	$K_{db}K_{myo}$
Urea, μmol/L	8388±1070	10 610±1053	8776±960	8017±527
Aspartic acid, μmol/L	9.7±0.5	11.0±0.8	7.6±1.1*	7.8±0.7*
Threonine, μmol/L	136.5±7.1	166.9±17.0	120.4±19.9	100.3±11.2*
Serine, μmol/L	102.8±4.7	119.4±11.9	89.5±11.4	71.3±7.3*
Asparagine, μmol/L	104.0±14.8	83.6±13.4	82.3±15.3	49.6±7.6†
Glutamic acid, μmol/L	48.4±2.9	45.5±8.6	45.0±7.7	40.4±4.5
Glutamine, μmol/L	598.7±42.6	547.5±22.4	496.8±63.5	486.0±34.3
Glycine, μmol/L	205.3±7.5	250.6±27.4	128.5±20.8*†	99.9±6.5*†
Alanine, μmol/L	387.0±38.9	424.3±76.2	403.4±62.2	261.7±34.6
Citrulline, μmol/L	48.2±3.9	41.2±3.3	49.9±8.0	37.8±5.4
Valine, μmol/L	200.4±24.3	199.6±13.2	218.2±30.7	213.7±38.5
Methionine, μmol/L	49.9±8.5	50.0±4.7	36.1±5.5	29.7±3.3
Isoleucine, μmol/L	93.9±11.9	91.8±7.6	108.0±14.3	101.8±21.8
Leucine, μmol/L	177.0±27.2	159.1±15.0	176.2±21.2	181.0±44.4
Tyrosine, μmol/L	66.2±13.6	67.0±5.2	56.8±10.4	49.6±7.9
Phenylalanine, μmol/L	69.5±8.2	71.8±6.7	68.5±7.1	74.6±9.0
Ornithine, μmol/L	45.8±5.4	34.0±5.1	97.3±20.9*	78.1±10.0
Lysine, μmol/L	246.7±36.6	230.8±29.7	177.1±24.9	156.5±14.7
1-Methyl-histidine, μmol/L	7.8±0.5	10.9±1.8	3.4±0.5*†	2.5±0.5*†
Histidine, μmol/L	64.7±7.9	59.4±5.6	57.7±8.1	49.7±7.2
Tryptophan, μmol/L	62.8±7.7	43.6±10.8	57.2±7.5	50.3±5.5
Arginine, μmol/L	79.5±13.3	72.6±8.6	17.2±6.5*†	17.6±3.2*†
Proline, μmol/L	62.2±2.9	55.0±11.5	52.0±8.0	47.3±4.5

Data are given as mean±SEM. N=5 to 12 for each group. H_{db} indicates heterozygous for the *db/db* leptin receptor mutation; H_{myo}, heterozygous for the myostatin gene; K_{db}, knocked out/mutated for the *db/db* leptin receptor mutation; K_{myo}, knocked out/mutated for the myostatin gene.
*$P \leq 0.05$ vs HK.
†$P \leq 0.05$ vs HH.

increased muscle mass, the $K_{db}K_{myo}$ mice have a significantly decreased amount of fat in the tissue and reduced free fatty acids and triglycerides within muscle itself.

Myostatin Deletion Improves Metabolic Status in Obese Mice

Baseline metabolic parameters and plasma characteristics are shown in Table 1 (whole body metabolism, plasma indexes of adiposity and insulin resistance, and indexes of oxidative stress and inflammation). Obese mice show a significant reduction in oxygen consumption and carbon dioxide production, and myostatin deletion does not improve either variable. Furthermore, voluntary activity (quantified with CLAMS) or daily activity (recorded with telemetry) shows similar patterns of total activity within both the lean and obese groups, with the obese group being severely and significantly reduced compared with controls. As described previously, increasing muscle mass is protective of hyperglycemic states, as indicated by lower plasma glucose and hemoglobin A1c levels and decreased glycosuria.[25] Lipemic changes were minor in obese mice and/or unaffected by myostatin deletion, with

only a minor reduction in plasma triglycerides being observed. Insulin levels remained elevated, implying continued hepatic insulin resistance.[28] Insulin-like growth factor levels were similar in all groups. Plasma indexes of inflammation (tumor necrosis factor-α) and antioxidants (interleukin-10 and uric acid) were unchanged with myostatin deletion. Adiponectin (Table 1, plasma hormones), known to be associated with hypertension, was unchanged between the lean and obese groups.[29,30] Plasma amino acid profiles (Table 2) showed no effect of myostatin deletion in the lean mice. Obesity depleted a limited number of amino acids, with or without myostatin, but the total concentration of amino acids was similar in all groups. Thus, augmentation of muscle mass produces a metabolic improvement (namely, glucose homeostasis) that is unrelated to generic improvements in insulin sensitivity or alterations in levels of activity in obese mice.

Myostatin Deletion Protects Against Obesity-Derived Hypertension

In vivo hemodynamics were obtained from radiotelemetry and shown in Figure 2. Mean arterial pressure was significantly
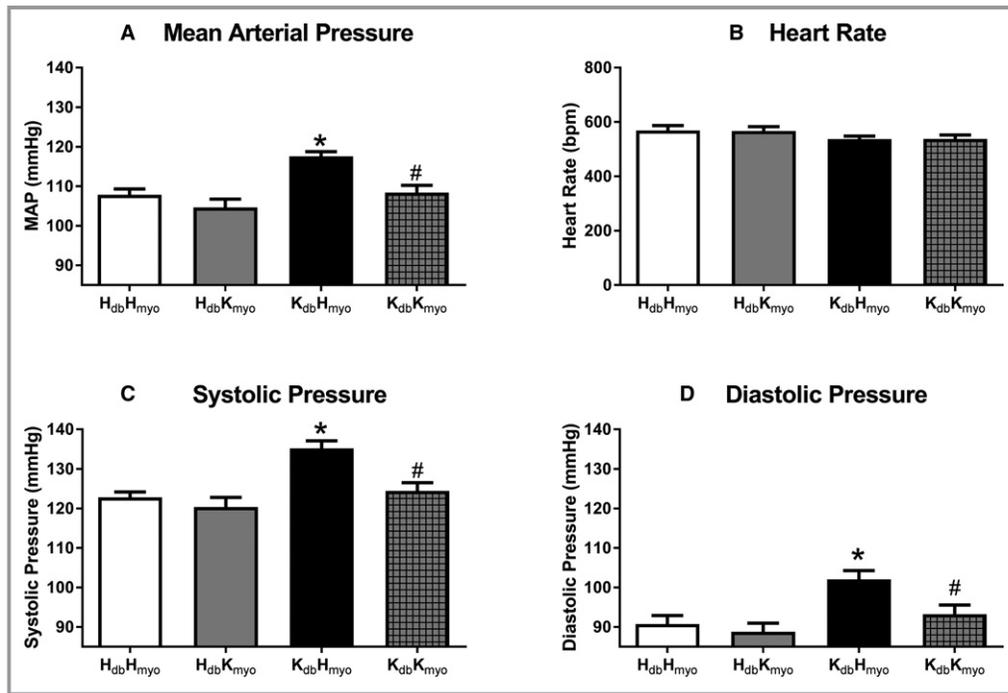

Figure 2. Myostatin deletion protects against obesity-derived hypertension. A, The mean arterial pressure (MAP) was recorded for 7 days using in vivo radiotelemetry in each group. The obese mice have a significant increase in MAP compared with the other groups. B, Heart rate was unchanged between any groups. Systolic (C) and diastolic (D) pressures were elevated significantly in the obese groups. N≥10 for each group. Bpm indicates beats per minute; H_{db}, heterozygous for the *db/db* leptin receptor mutation; H_{myo}, heterozygous for the myostatin gene; K_{db}, knocked out/mutated for the *db/db* leptin receptor mutation; K_{myo}, knocked out/mutated for the myostatin gene. *$P \le 0.05$ vs HH; #$P \le 0.05$ vs KH.

elevated in $K_{db}H_{myo}$ mice (Figure 2A). Myostatin deletion rescues obesity-derived hypertension back to levels of control. Heart rate (Figure 2B) remains unchanged between the 4 groups. Systolic (Figure 2C) and diastolic (Figure 2D) pressure demonstrated parallel elevations with obesity, which are reduced with myostatin deletion. Circadian rhythms remained unchanged between groups, with nocturnal dipping continuing to be blunted with obesity and unchanged with myostatin deletion ($H_{db}H_{myo}$, 9.1±1.2 Δmm Hg; $H_{db}K_{myo}$, 9.7±2.3 Δmm Hg; $K_{db}H_{myo}$, 2.5±0.7 Δmm Hg; $K_{db}K_{myo}$, 3.8±1.4 Δmm Hg).

Myostatin Deletion Alters Fluid Dynamics in Obese Mice

Metabolic cages were used to assess food intake, water consumption, and urine production (Figure 3A through 3C). Food intake was significantly increased with the obese groups, and myostatin deletion did not alter food consumption, explaining similar levels of obesity and adiposity (Table 1). However, the polydipsia (Figure 3B) and polyuria (Figure 3C) that was observed in the $K_{db}H_{myo}$ mice was significantly improved with myostatin deletion ($K_{db}K_{myo}$). Analysis of the electrolytes in urine showed that myostatin

deletion results in a significant increase in the concentration of sodium in the urine in both lean and obese mice (Figure 3D). When normalized to urine output (Figure 3E), it is observed that the total daily excretion is the same between the lean and obese groups, confirming sodium balance. Microalbuminuria, a measure of kidney injury and expressed as the albumin/creatinine ratio in Figure 3F, was assessed in all groups. Obesity ($K_{db}H_{myo}$) significantly increased microalbumin production, and this was improved with myostatin deletion ($K_{db}K_{myo}$). This improvement in fluid balance is independent of changes in aldosterone and vasopressin (Table 1, plasma hormones).

Myostatin Deletion Normalizes Changes in Blood Pressure With Dietary Salt

Renal fluid dynamics, sodium excretion, and blood pressure were measured in response to short-term changes (3 days) in dietary salt (Figure 4 and Table 3). Lean and obese control mice experienced modest elevations in blood pressure with the high-salt diet and corresponding increases in salt excretion. High salt loading eliminates the significant improvements observed at baseline with blood pressure and kidney function in the obese myostatin knockout mice. However,
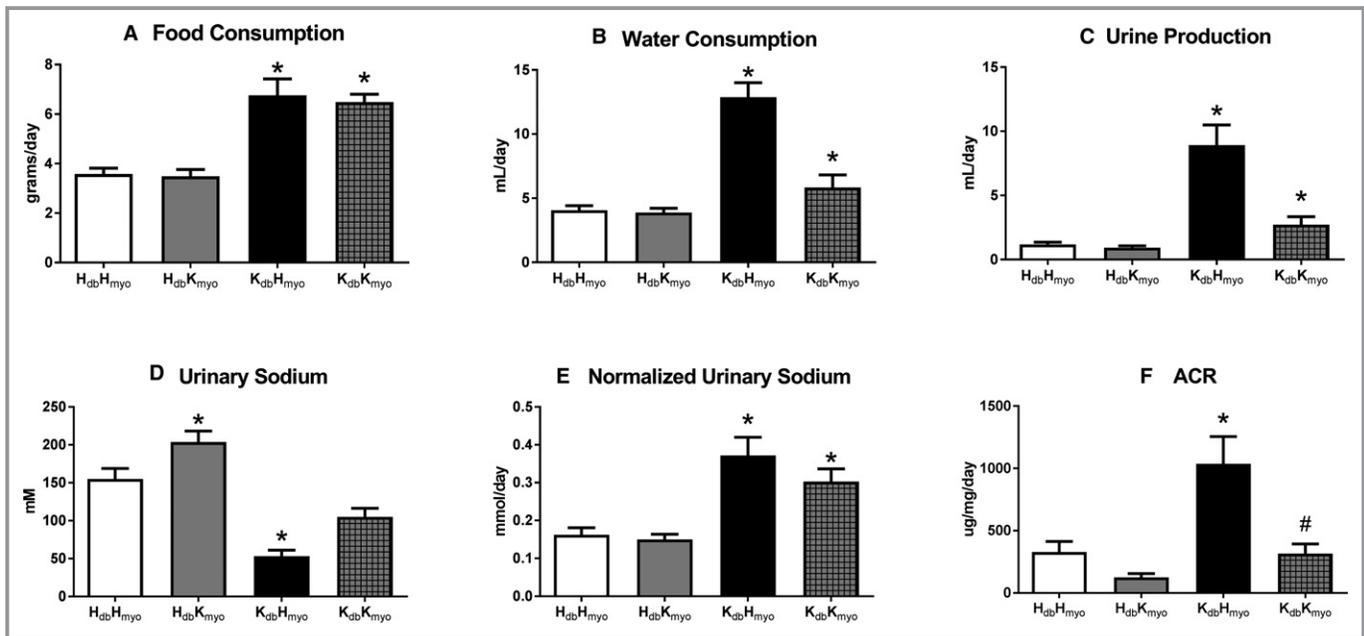

Figure 3. Myostatin deletion improves fluid balance in the obese mouse. Despite similar food intake (A) between the lean and obese groups, the obese myostatin knockout mice consumed significantly less water (B) and excreted less urine (C). D, Myostatin deletion allowed for sodium to be concentrated in the urine. E, When normalized to urine output, the daily sodium excretion was similar intergroup, although significantly increased in the obese groups (N>9). F, The albumin/creatinine ratio (ACR), a clinical measure of kidney injury, was measured for each group (N=6), and the obese control had a significantly elevated ACR. N≥10 for each group. H_{db} indicates heterozygous for the *db/db* leptin receptor mutation; H_{myo}, heterozygous for the myostatin gene; K_{db}, knocked out/mutated for the *db/db* leptin receptor mutation; K_{myo}, knocked out/mutated for the myostatin gene. *$P \leq 0.05$ vs HH; #$P \leq 0.05$ vs KH.

myostatin deletion in lean mice continued to provide significant protection against increases in blood pressure and improved sodium excretion. Low-salt diet normalized blood pressure and kidney function in obesity to levels of control in all groups.

Myostatin Deletion Protects Against NOX4 Increases in the Kidney

Reverse transcription–polymerase chain reaction was used to quantify the NOXs in total kidney homogenates (Figure 5), and primers are shown in Table 4. Figure 5A shows that obesity ($K_{db}H_{myo}$) significantly increases NOX4 in the kidney and that myostatin deletion significantly reduces NOX4 expression in obesity ($K_{db}K_{myo}$). NOX1 and NOX2, known to play a role in kidney function, were measured, and no intergroup differences were observed (Figure 5B and 5C). The primary oxidant produced by NOX4 is hydrogen peroxide, which is significantly elevated in concentration and daily excretion in the urine of obese

mouse (Figure 5D and Table 1, indexes of oxidative stress and inflammation) and significantly reduced with myostatin deletion.

Discussion

The current study provides the first test of the hypothesis that muscle mass modulates hypertension and renal dysfunction in obesity without attendant weight loss. We confer one of the benefits of exercise, increased muscle mass, onto obese mice via deletion of myostatin. We present 2 novel findings from this study: (1) increasing and/or restoring skeletal muscle mass is a mechanism to improve hypertension in obesity, and (2) the mechanism of this improvement likely reflects renal protection from the consequences of obesity, resulting in reduced expression of oxidant enzymes, glycosuria, and albuminuria.

Obesity remains the primary determinant of cardiovascular disease in the US population, with little improvement in sight. Exercise has been shown to limit cardiovascular disease in
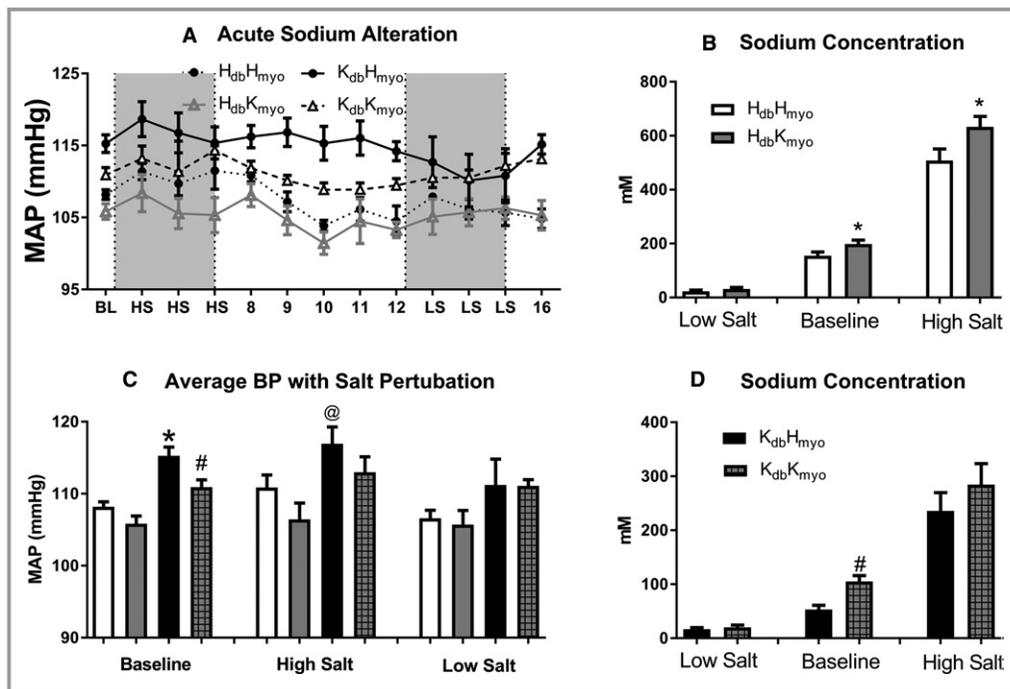

Figure 4. Myostatin deletion normalizes changes in blood pressure because of dietary salt. A, The time course of mean arterial blood pressure (MAP) in all groups at baseline (BL; 3-day average) and under short-term (3 days) high-salt (HS) and low-salt (LS) conditions. B, The 3-day average of blood pressure under B, HS, and LS is shown. HS diet produced modest increases in blood pressure in all groups, except heterozygous/knocked out/mutated (HK). LS diet lowered the KH blood pressure to levels of control. N≥7 for obese controls, and N≥4 for remaining groups. The sodium concentration in urine under all conditions is shown for the lean mice (C) and the obese mice (D). N≥7 for lean mice in C, and N≥5 for obese mice in D. H_{db} indicates H for the *db/db* leptin receptor mutation; H_{myo}, H for the myostatin gene; K_{db}, K for the *db/db* leptin receptor mutation; K_{myo}, K for the myostatin gene. *$P \leq 0.05$ vs HH; #$P \leq 0.05$ vs KH; @$P \leq 0.05$ vs HK.

obese individuals, but many individuals, because of age or excess weight, cannot exercise at a level that would confer cardiovascular benefit. The mechanisms by which exercise affects cardiovascular function, especially in obese populations, are poorly understood, but if the benefits of exercise could be replicated pharmacologically, new therapies could potentially emerge.

Exercise has been long documented to improve blood pressure in obese individuals, even in the absence of weight loss.[31–33] In 2004, studies from our institution indicated that blood pressure control, specifically recovery of poststress pressor increases, was correlated with high lean body mass in overweight subjects, arguing the skeletal muscle was an unappreciated determination of blood pressure control.[34] Previous work from our group showed that myostatin deletion in obese mice augmented muscle mass, improved glucose clearance, and increased endothelial NO bioavailability in the mesenteric microvasculature, largely by blunting increases in vascular NOX1.[8,25] Given that myostatin deletion in obesity protects against obesity-induced endothelial dysfunction, the current study sought to extend that observation by determining if improvements to vascular health would positively affect blood pressure. Although this increase in muscle mass had no effect on blood pressure in lean mice, it prevented the increase seen in obese mice as either mean systolic or diastolic pressure. More important, this study ruled out key variables and potential confounders that could affect blood pressure. Weight loss,

loss of adiposity, and increases in activity are known to have positive effects on blood pressure profiles.[35–39] However, the experimental paradigm used in the current study uses obese mice that share similar metabolic profiles in that body mass, total fat content, oxygen consumption, and activity levels remain unchanged with or without increased muscle mass. Furthermore, although glucose homeostasis is improved in our obese knockout mice, additional plasma indexes of insulin resistance remained consistent (leptin, insulin, and insulin-like growth factor 1). Thus, it is unlikely that the observed cardiovascular improvement is a reflection of nonspecific effects of myostatin on body weight but a more specific relationship between glucose metabolism, vascular and renal function, and blood pressure. This work is supported by Jiang et al, who showed that adenoviral inhibition of myostatin increased muscle mass and improved glucose homeostasis in the *db/db* mouse, independent of changes in weight.[40]

Although increased muscle mass may provide a potential mechanism for the effects of exercise on blood pressure, the mechanistic links between blood pressure and muscle mass require deeper study. In the current study, several simple explanations are ruled out. Aldosterone and vasopressin levels and GFR were similar in all groups, whereas tumor necrosis factor-α levels were elevated in obese mice but not lowered by hypermuscularity, suggesting similar degrees of whole body inflammation (Table 1). Further quantification of the antioxidant profile (interleukin-10) showed no changes between groups.

Table 3. Metabolic Characteristics Under Salt Perturbation

Variable	$H_{db}H_{myo}$	$H_{db}K_{myo}$	$K_{db}H_{myo}$	$K_{db}K_{myo}$
Additional baseline indexes				
Potassium, mmol/L	419.6±31.3	448.6±35.0	135.9±25.6*	264.3±41.2*
Normalized potassium, mmol/d	0.408±0.042	0.340±0.037	0.812±0.081*	0.666±0.098*
Chloride, mmol/L	333.6±25.3	392.3±38.8	90.4±13.8*	195.3±26.4*
Normalized chloride, mmol/d	0.315±0.030	0.278±0.031	0.622±0.074*	0.503±0.075*
High salt				
Food consumption, g/d	2.6±0.3	3.4±0.3	7.1±1.2	7.1±1.8
Water consumption, mL/d	6.1±0.8	7.6±0.8	18.0±3.0	15.0±3.1
Urine production, mL/d	2.5±0.3	2.6±0.3	17.2±2.0	12.5±2.9
Low salt				
Food consumption, g/d	2.1±0.3	2.6±0.2	6.2±0.8	6.7±0.9
Water consumption, mL/d	4.8±1.1	4.0±0.7	6.9±1.6	6.7±1.3
Urine production, mL/d	1.9±0.5	0.9±0.4	4.6±0.8	4.6±1.5

Data are given as mean±SEM. N=5 to 12 for each group. H_{db} indicates heterozygous for the *db/db* leptin receptor mutation; H_{myo}, heterozygous for the myostatin gene; K_{db}, knocked out/mutated for the *db/db* leptin receptor mutation; K_{myo}, knocked out/mutated for the myostatin gene.
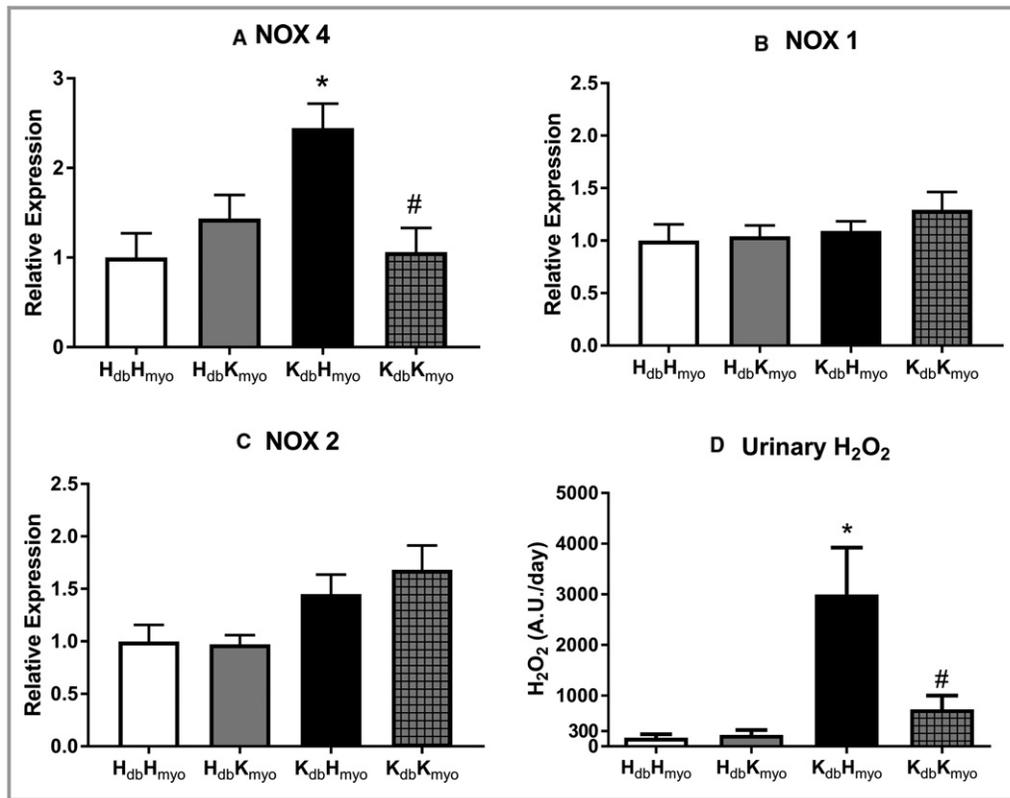*$P\leq0.05$ vs HH.

Figure 5. Myostatin deletion prevents increases in nicotinamide-adenine dinucleotide phosphate, reduced form, oxidase 4 (NOX4) in renal tissue and decreases urinary hydrogen peroxide. NOX 1, 2, and 4 were quantified in total kidney homogenates of all groups. A, Obesity significantly upregulates NOX4 in the kidney (knocked out/mutated/heterozygous [KH] group) compared with the lean control (HH group). Myostatin deletion significantly inhibits NOX4 upregulation in obesity (KK group). NOX1 and NOX2 were unchanged between groups (Panels B and C). D, Daily excretion of urinary hydrogen peroxide was assessed and found to be significantly elevated with obesity and reduced with myostatin deletion. N=6 to 10 for each group. H_{db} indicates H for the *db/db* leptin receptor mutation; H_{myo}, H for the myostatin gene; K_{db}, K for the *db/db* leptin receptor mutation; K_{myo}, K for the myostatin gene. *$P\leq0.05$ vs HH; #$P\leq0.05$ vs KH.

Three potential mechanistic insights were obtained in the current studies that may shed light on how muscle mass improves blood pressure regulation by protecting the kidney.

Table 4. Primer Sequences

Name	Sequence
NOX 1 F	ATGGTAACGTCAGCTATGGA
NOX 1 R	ATTGTGAGGATGGCTTTCAC
NOX 2 F	TGTTTTCATTTCCTCATCAGAAG
NOX 2 R	GAGAGTCCTTCATTCACAGC
NOX 4 F	AATGTTGGGCCTAGGATTGT
NOX 4 R	TTCACTGAAAAGTTGAGGGC
GAPDH F	CCCTTAAGAGGGATGCTGCC
GAPDH R	TACGGCCAAATCCGTTCACA

F indicates forward; NOX, nicotinamide-adenine dinucleotide phosphate, reduced form, oxidase; R, reverse.

First, the kidney is protected from hyperglycemia. Increases in blood glucose can impair functions of the glomerulus (filtration, permeability, and renal regulation of blood pressure) and drive fluid and sodium loss when glucose exceeds ≈150 mg/dL.[41–44] In the current study, as shown previously, myostatin deletion increases muscle mass and reduces plasma glucose levels significantly.[25] Moreover, glycosuria is resolved by hypermuscularity in obese mice. Because glucose is filtered freely, a decrease in glycosuria indicates that glucose in the glomerulus has decreased below a level that cannot be reabsorbed and is thus likely renoprotective.

Second, the kidney is protected from fluid excess. Processing of large fluid volumes (polydipsia and polyuria) has been well documented in obese rodents and humans, usually a reflection of a diabetic phenotype.[45–47] In the current study, fluid intake and excretion are markedly reduced by deletion of myostatin, especially in obese mice. Functionally, the result was urine in which sodium was more concentrated. This concentration was evident even in lean

mice when sodium intake was increased, indicating the muscle mass has effect on whole body fluid balance even in the absence of obesity. It is not entirely clear from these studies what the precise site of action is from the muscle's effects (ie, does it decrease fluid intake, thereby reducing the need to produce urine, or produce a more concentrated urine, thereby reducing thirst).[48,49] Moreover, the protection from hypertension was evident in lean mice even in the presence of elevated salt intake (although a low salt intake predictably eliminated differences in blood pressure among groups). Thus, although salt effects are mild and causes of changes in fluid balance are unresolved, it can be concluded that the fluid load on the kidney is reduced by increases in muscle mass.

Finally, the kidney is protected from excess oxidant enzymes. In metabolically compromised animals, oxidant stress is thought to be an important link between glucose disturbances and impaired function of the kidney. Several reports have described a causal role for NOX4 in renal dysfunction that can lead to hypertension.[50,51] In salt-sensitive rats, urinary hydrogen peroxide has been shown to be elevated with increased renal perfusion pressure, and deletion of renal NOX4 attenuates hypertension.[52–54] In our obese mouse model, NOX4 expression is elevated in renal tissue and urinary hydrogen peroxide is increased in both concentration and daily excretion, whereas expression of other NOX isoforms was unchanged. This increase is resolved by deletion of myostatin, suggesting that a mechanistic link between muscularity and lower blood pressure may be reductions in NOX4.

Taken together, the experiments described in this study provide the first evidence that increased muscle mass can reduce blood pressure in obesity, independent of changes in body weight or adiposity. This improvement correlates with improved glucose tolerance and renoprotection, as evidenced by improved albuminuria. The conclusion of parsimony is that increased muscle mass absorbs excess glucose and reduces fluid intake, unloading the kidney and reducing NOX4-mediated oxidant stress. This may prevent the progression of renal injury evident in metabolically compromised states, which ultimately leads to hypertension. Further study of the specific components of this process, especially sources of renal oxidant stress in obesity, is an important next step in understanding this process.

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Disclosures

None.

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