Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems

Sherry O. Kasper,1 Christy S. Carter,1 Carlos M. Ferrario,1 Detlev Ganten,2 Leon F. Ferder,3 William E. Sonntag,1 Patricia E. Gallagher,1 and Debra I. Diz1

1Hypertension and Vascular Disease Center and Physiology and Pharmacology Department, Wake Forest University School of Medicine, Winston-Salem, North Carolina; 2Charite, University Medicine Berlin, Berlin, Germany; and 3Department of Physiology and Pharmacology, Ponce School of Medicine, Ponce, Puerto Rico

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PREVIOUS STUDIES indicate lower body weight in mice with systemic angiotensinogen deficiency (42), body weight differences in patients with polymorphisms of the angiotensinogen gene (12, 57), and overexpression of angiotensinogen in adipose tissue of obese Zucker rats (30) and human subjects (26). Further characterization of indexes of growth and metabolism in ASrAoegen rats compared with (mRen2)27 and SD rats, which express elevated versus normal brain and tissue angiotensin II levels, respectively, revealed that serum leptin was 100–200% higher in SD and (mRen2)27 rats at 46 wk and 69 wk of age. Consistent with low serum leptin, ASrAoegen rats had higher food intake (73%) compared with SD or (mRen2)27 rats. (mRen2)27 rats had higher resting insulin levels than ASrAoegen rats at all ages. Insulin levels were constant during aging in (mRen2)27 rats. (mRen2)27 rats had higher resting insulin levels than ASrAoegen rats, whereas an increase occurred in SD rats, leading to higher insulin levels at 46 and 69 wk of age compared with ASrAoegen rats. IGF-1 was comparable among strains at all ages, but (mRen2)27 rats had longer and ASrAoegen rats had shorter tail lengths versus SD rats at 15 wk of age. In conclusion, reduced expression of glial angiotensinogen blunts the age-dependent rise in insulin levels and weight gain, findings that mimic the effects of long-term systemic blockade of the RAS or systemic knockout of angiotensinogen. These data implicate glial angiotensinogen in the regulation of body metabolism as well as hormonal mechanisms regulating blood pressure.

insulin resistance; metabolic syndrome

METHODS

Animals. Rats from all three lines [Hannover SD, heterozygous (mRen2)27, or ASrAoegen(680)] (ASrAoegen rats); high brain and tissue ANG II [(mRen2)27 rats]; or control Hannover SD rats] to gain insight on the influence of brain ANG II on overall metabolism.

PREVIOUS STUDIES indicate lower body weight in mice with systemic angiotensinogen deficiency (42), body weight differences in patients with polymorphisms of the angiotensinogen gene (12, 57), and overexpression of angiotensinogen in adipose tissue of obese Zucker rats (30) and human subjects (26). Blockade of the renin-angiotensin system (RAS) is known to lower blood pressure associated with hypertension, and, recently, angiotensin-converting enzyme (ACE) inhibitors (1) and angiotensin II (ANG II) type 1 (AT1) receptor blockers (14, 35) have been shown to improve insulin insensitivity in long-term studies. Treatment of obese Zucker rats with irbesartan improved whole body insulin sensitivity and glucose uptake into the soleus and epitrochlearis muscle after 21 days of treatment (33), due in part to an increase in glucose transporter 4 (Glut4) protein levels. Rats with overexpression of tissue RAS, including the brain, show insulin resistance as early as 6 wk of age (32).

During aging, increases in body weight gain and insulin resistance develop in concert with increases in blood pressure in Sprague-Dawley (SD) rats (11, 45). Rats treated long term with enalapril exhibit a lower body fat mass compared with lean mass (9), and smaller increases in body weight occurred in Fisher 344 rats treated for 1 yr with L-158,809, an AT1 receptor blocker in association with lower insulin and leptin levels and without significant differences in blood pressure (27). This suggests that interruption of the RAS, not just lowering of blood pressure, is important in improving insulin sensitivity and regulating body weight. Mechanisms involved in these long-term effects may include alterations in insulin signaling in skeletal muscle, because short-term ANG II infusions reduce this component (23, 24). However, it is not known from these studies whether the effects of ANG II are at central or peripheral sites, because long-term treatments with either AT1 antagonists or ACE inhibitors should access brain sites (6, 28, 39, 46).

Interestingly, specific replacement of brain ANG II in mice with systemic knockout of angiotensinogen corrects renal and other deficits occurring in these mice (40), although indexes of body metabolism were not studied. The brain areas involved in mediating autonomic actions of the RAS and expressing AT1 receptors are involved in regulating circulating insulin and leptin levels as well as food intake and body energy metabolism (20, 44, 47). For these reasons, we used three strains of rats with varying amounts of the brain RAS to assess indexes of body growth and metabolism [low glial angiotensinogen, ASrAoegen(680)] (ASrAoegen rats); high brain and tissue ANG II [(mRen2)27 rats]; or control Hannover SD rats] to gain insight on the influence of brain ANG II on overall metabolism.
Radioimmunoassay and Northern blot hybridization. Nonfasting leptin and insulin were measured by radioimmunoassay (Linco Research; St. Charles, MO). Glucose was measured using a Freestyle Glucose Analyzer. A subset of glucose measurements was made on both a Bayer Advia 1650 general analyzer and the Freestyle Glucose Analyzer to ensure accurate values ($r^2 = 0.95$). All measurements were made on the Freestyle Glucose Analyzer for all animals. The IGF-1 assay was performed as previously described (56). Materials for analysis of IGF-1 were the generous gift of Dr. A. Parlow and the National Hormone and Peptide Program.

RNA was isolated from the brain, adipose tissue, and pancreas for the detection of the antisense oligonucleotide to angiotensinogen using the TRIzol method according to the manufacturer’s instruction. RNA aliquots were incubated with RQ1 DNase to degrade any residual contaminating genomic DNA. The RNA concentration was quantified by ultraviolet spectroscopy, and degradation was assessed by ethidium bromide staining intensity of 28S and 18S rRNA after agarose gel electrophoresis. Northern blot hybridization was performed as previously described (25) using $^{32}$P-labeled antisense cRNA as a probe (53).

Tail length. Tail length, as a measure of growth (29), was measured using a standard metric ruler from the anus of the animal to the tip of tail by the same experimenter to ensure uniformity.

Blood pressure. Blood pressure was measured in all groups of animals using the tail-cuff method (4, 19). At least five determinations were made in each animal and averaged for a single determination per session. Rats were not trained to the tail cuff procedure but were trained to handling by the same investigators who performed the tail-cuff procedures.

Statistics. Two-way ANOVA was used to assess overall interactions across strain and ages (3 x 3 design). Subsequently, one-way ANOVA and Student-Newman-Keuls post hoc tests were used to compare each genotype or age to each other.

RESULTS

Blood pressure, heart rate, body weight, and heart weight measurements. As shown in Fig. 1A, there was an effect of age ($F = 15.15, 2; P < 0.0001$) and genotype ($F = 155.8, 2; P < 0.0001$) on blood pressure by two-way ANOVA. At the 15-wk time point, blood pressure was highest in the (mRen2)27 rats and lowest in the ASrAogen rats, consistent with other studies (17–19, 53, 54). In SD rats, blood pressure was highest at 69 wk compared with 15 and 46 wk, consistent with an age-related increase in systolic pressure as reported previously in (17–19, 53, 54). In SD rats, blood pressure was highest at 69 wk compared with (mRen2)27 animals but lower than ASrAogen animals using the tail-cuff method (4, 19). At least five determinations across strain and ages (3, 34, 37). The blood pressure at 69 wk was lower than that at the 15- and 46-wk time points in the ASrAogen animals. (mRen2)27 rats had a decline in blood pressure over time. This was accompanied by a larger heart-to-body weight ratio compared with either the SD or ASrAogen rats (Fig. 1B). There was also an effect of genotype ($F = 56.75, 2; P < 0.0001$) and age ($F = 7.833, 2; P = 0.0008$) and an interaction ($F = 6.731, 4; P = 0.0001$) on the heart-to-body weight ratio. Upon necropsy, fluid was found in the chest cavity of 69-wk-old (mRen2)27 animals, suggesting the presence of heart and renal failure as well as necropsy reports suggesting heart failure with pulmonary congestion and renal failure with protein loss. There was a significant main effect of genotype for heart rate ($F = 32.14, 2; Fig. 1C$). The heart rate in ASrAogen animals tended to be lower at 15 wk ($P = 0.06$) compared with SD and (mRen2)27 animals and was significantly lower at both 46 and 69 wk ($P < 0.05$). SD animals had a similar heart rate at the 15-wk time point compared with (mRen2)27 but lower compared with this strain at 46 and 69 wk. ASrAogen rats had a significantly lower body weight than either the SD or (mRen2)27 rats at all ages (Table 1).

Serum insulin, leptin, glucose, and IGF-1. There was an effect of genotype ($F = 7.971, 2; P < 0.05$) and a significant interaction for genotype and age ($F = 2.957, 4; P < 0.05$) on insulin. As shown in Fig. 2A, serum insulin levels were lower in ASrAogen animals compared with (mRen2)27 animals at 15 wk and both other groups at 69 wk. SD animals had higher insulin at 69 wk compared with the other groups. There was a significant effect for both genotype ($F = 4.323, 2; P < 0.05$) and age ($F = 3.765, 2; P < 0.05$) on serum glucose levels. Serum glucose, as shown in Fig. 2B, was also lower in ASrAogen animals at 15 wk compared with (mRen2)27 rats,
Table 1. Body weight for ASrAogen, SD, and (mRen2)27 rats

<table>
<thead>
<tr>
<th>Age</th>
<th>ASrAogen</th>
<th>SD</th>
<th>(mRen2)27</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 wk</td>
<td>442±9*(14)</td>
<td>551±21† (13)</td>
<td>599±5‡ (12)</td>
</tr>
<tr>
<td>46 wk</td>
<td>326±7 (6)</td>
<td>386±6 (7)</td>
<td>392±6 (12)</td>
</tr>
<tr>
<td>69 wk</td>
<td>459±20* (16)</td>
<td>589±15* (6)</td>
<td>621±15* (5)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; numbers in parentheses indicate number of animals. SD, Sprague-Dawley. *P < 0.001 vs. 15-wk-old ASrAogen rats; †P < 0.001 vs. 46-wk-old ASrAogen rats; ‡P < 0.001 vs. 69-wk-old ASrAogen rats.

and, at 69 wk, serum levels of glucose were higher in SD compared with (mRen2)27 animals. There was a significant effect of genotype (F = 12.28, 2; P < 0.05) on serum leptin. Leptin levels in serum were similar among groups at the early time point but were lower in ASrAogen animals compared with SD and (mRen2)27 animals at both 46- and 69-wk time points (Fig. 3A). Finally, IGF-1 was comparable in the three strains of animals at all ages (Fig. 3B).

Food intake and tail length. There was a significant effect of genotype (F = 32.04, 2; P < 0.05) and age (F = 14.67, 2; P < 0.05) on food intake. Food intake was higher in ASrAogen animals compared with the other groups at all ages (Fig. 4A).

Also, there was a significant effect of genotype (F = 31.86, 2; P < 0.05) and age (F = 26.12, 2; P < 0.05) on tail length, an indicator of growth (29). Tail length was shorter in ASrAogen animals compared with the other groups at all ages (Fig. 4B). On visual inspection of the animals, there was also less visceral fat in ASrAogen animals.

Northern blot analysis. Northern blot hybridization was performed to assess the expression of angiotensinogen antisense RNA in the brain, adipose, and pancreatic tissue from transgenic rats. A strong hybridization signal was detected with RNA isolated from brain tissue of the transgenic rats; however, no band was observed with pancreatic or adipose RNA (3 separate experiments with tissue collected from 3 animals). This result is consistent with the tissue-specific expression of antisense mRNA in the brain, as originally reported in these rats (53).

DISCUSSION

ASrAogen animals have low resting blood pressure and insulin and glucose levels, associated with hyperphagia and reduced growth, compared with (mRen2)27 rats at 15 wk of age and low blood pressure, insulin, and leptin at the 69-wk time point compared with both SD and (mRen2)27 animals, accompanied by a lower body weight at all ages compared with
Mechanisms involved in these long-term effects may include alterations in insulin sensitivity and glucose utilization in the peripheral circulation. AT1 receptor blockers in a model of Type II diabetes (the obese Zucker rat) improve insulin resistance and overall insulin sensitivity. This improvement is partially due to an increase in the insulin-dependent glucose transporter Glut4 in the heart, soleus, and plantaris muscles (33). KK-Ay mice, a model for Type II diabetes, treated with valsartan had a profile similar to the treated Zucker rats with improved insulin sensitivity and increased Glut4, which was associated with an increase in insulin-induced phosphorylation of insulin receptor substrate-1 (IRS-1) and coupling of IRS-1 with the p85 subunit of phosphatidylinositol 3-kinase in skeletal muscle as well as an increase in protein levels (55). In Fisher-344 rats that do not develop hypertension as they age, long-term AT1 receptor blockade was associated with lower body weight and lower insulin and leptin levels after 1 yr of treatment relative to untreated animals (27). Acute and chronic administration of an ACE inhibitor in patients decreases the daily glucose profile and improves insulin sensitivity in Type II diabetic patients (32). However, there are contradictory reports showing that ANG II improves insulin sensitivity (51). Moreover, it is not known from previous studies whether the effects of ANG II are at central or peripheral sites.

In this study, ASrAogen animals had similar serum insulin levels compared with SD animals at 15 wk, but levels were higher in (mRen2)27 rats. Rats with inhibition of glial angiotensinogen expression failed to show a rise in serum insulin and glucose levels at the 69-wk time point compared with SD animals. In fact, ASrAogen animals have low levels of serum insulin at 69 wk compared with both SD and (mRen2)27 animals. The higher serum insulin and glucose in SD animals suggest insulin resistance at the older time point. (mRen2)27 rats were shown to be insulin resistant at 6 wk of age, with an impaired insulin response to the oral glucose tolerance test and impaired insulin-mediated glucose transport into the epitrochlears and soleus muscle compared with SD animals (38). The improvement in insulin sensitivity in these rats after long-term treatment with an AT1 antagonist was related to reductions in oxidative stress (5). With the use of the oral glucose tolerance test, preliminary reports have shown that ASrAogen rats have a low and (mRen2)27 rats a high glucose-insulin index with SD rats intermediate at 16 wk of age, with the development of insulin resistance in older SD rats to a level comparable to that of (mRen2)27 rats (48). Thus, regardless of the mechanism, ASrAogen animals do not exhibit the aging-related increase in serum insulin experienced by SD animals. Moreover, the data from these various reports imply that glial-derived ANG II may be an important factor in both aging and overall body metabolism independent of age and blood pressure.

There was also a lower growth rate, as evidenced by the shorter tail length, in ASrAogen animals compared with the other groups. This difference was dependent on the genotype of the animals because there was a difference between not only ASrAogen rats and the other groups but also between SD and (mRen2)27 rats. However, the difference in growth was not due to a difference in IGF-1 and, therefore, not due to a difference in growth hormone, because these levels were similar for all groups at all ages. Although the difference in growth may underlie the difference in body weight, the contribution of reduced fat mass likely also plays a role (11), because there
was noticeably less visceral fat in ASrAogen rats, as is observed in rats treated long term with enalapril (9). Although the actions of exogenous ANG II given centrally on overall body weight are not clear (10), there was a clear effect of genotype on body weight and tail length in this study at the early time point. Body weight differences diverge with age to exhibit an effect of genotype during aging as well. However, these studies further suggest that “normal” brain ANG II levels with age, not necessarily elevated levels, are important in the increase in body weight because there is no difference between SD and (mRen2)27 animals at 69 wk. Because there is elevated expression of the mouse renin gene in a variety of tissues in (mRen2)27 animals (2, 49, 52, 54), we cannot rule out an effect of adipose tissue or other peripheral tissue RAS components in this effect in these animals. However, in ASrAogen rats, there was no expression of the antisense mRNA to angiotensinogen detected in adipose or pancreatic tissue, suggesting that in these animals the metabolic effect is not due to a local effect of the antisense mRNA. If there is any reduction in the adipose or pancreatic tissue RAS, it would appear to be secondary to the glial deficiency of angiotensinogen.

Serum leptin levels were similar at the 15-wk time point among the three groups but were lower in ASrAogen rats compared with SD and (mRen2)27 rats at the later two time points. This was an expected result because leptin is released from adipose tissue (41) and there was a large difference in body weight among the groups, especially at these later ages. Because leptin is also an appetite suppressor (41), the low leptin levels would be consistent with the fact that ASrAogen animals had a higher food intake than the other groups at all ages. However, this difference in food intake was noted even at 15 wk, when there was no difference in circulating leptin. Therefore, the difference in body weight was not simply due to a suppression of appetite in ASrAogen animals and may be interpreted as the result of a central-mediated long-term effect of ANG II on overall body metabolism. The lower leptin level is also consistent with a lower sympathetic nervous system outflow in ASrAogen rats, as evidenced by the lower resting blood pressure and lower heart rate reported here.

AT1 receptors are present in the hypothalamic areas involved in feeding behaviors and the release of hormones concerned with satiety and energy metabolism (20, 44, 47) and are elevated in older ASrAogen rats (36, 43). Interactions have been reported for leptin and insulin with cholecystokinin and neuropeptide Y, factors influencing both satiety and blood pressure regulation, within the dorsomedial medulla oblongata and vagal sensory and motor systems, pathways known to exhibit high densities of ANG II receptors (7, 18). Low plasma vasopressin would also be consistent with an impaired release of insulin from pancreatic β-cells in response to a glucose load (50). Because ASrAogen rats exhibit a mild form of diabetes insipidus as a result of low circulating and brain vasopressin (8, 53), a variety of factors may influence the dynamic secretion of insulin and leptin in these rats as a direct result of alterations in RAS-dependent brain pathways.

The characteristics demonstrated by ASrAogen animals are similar to mice overexpressing human uncoupling protein 3 (UCP-3). UCP-3 is a protein involved in heat production due to inefficient use of glucose at the mitochondrial level. These mice have hyperphagia, reduced weight compared with the wild type, and lower insulin and glucose levels (13). Therefore, UCP proteins in the brain (16) may be a target of RAS-dependent pathways. ACE inhibitors can increase the number of mitochondria in the liver and heart (21, 22), which may cause an increase in oxygen consumption and glucose utilization, and ACE inhibitors and AT1 blockers influence mitochondrial function during aging (3, 15). However, there is no direct link between the brain RAS and the effects in mitochondria at the present time, indicating that the effects of ACE inhibitors or AT1 blockers on energy metabolism may be distinct from the mechanisms mediating the apparent improved energy metabolism in animals with low glial angiotensinogen.

In the present study, rats with varying levels of brain angiotensins were examined for indexes of growth and metabolism. One might expect a “dose effect” of the low, normal, and high RAS in the three strains if the RAS is the major contributor to the variables studied. In this study, we did indeed see an effect of genotype on insulin, glucose, food intake, and tail length as well as blood pressure and body weight with ASrAogen rats at the low end and (mRen2)27 rats at the upper end of the normal relationship expressed in SD rats. The graded effect on certain variables lacking at the early time point was apparent at the later time point (leptin); a finding interpreted as providing evidence for a role for brain RAS in the events associated with aging rather than direct regulation of that component under normal resting conditions. However, certain features of (mRen2)27 rats did not follow a parallel pattern consistent with a directly opposite effect to ASrAogen rats in all aspects studied at all time points. This is not surprising because these animals develop severe cardiovascular damage in the heart, kidneys, and brain at early time points in the aging process that may secondarily alter the overall response. In addition, whereas ASrAogen rats appear to have a targeted disruption of the glial RAS, the overexpression of RAS in (mRen2)27 rats extends not only in the brain to neuronal and glial pathways but to peripheral vascular tissues as well.

These data reveal that rats with low glial ANG II are protected from many features of the metabolic syndrome, including weight gain, a rise in blood pressure, and insulin resistance as they age. SD animals, on the other hand, exhibit signs of developing the metabolic syndrome, including a rise in systolic blood pressure and high serum insulin and glucose at the 69-wk time point compared with both 15 and 46 wk, suggesting that brain ANG II plays an important role in the development of many of the components associated with the metabolic syndrome.

**Perspectives.** The metabolic syndrome, characterized by insulin resistance, hyperinsulinemia, dyslipidemia, central adiposity, and hypertension (58), has become an epidemic in the United States (31). Blockade of the RAS is known to lower blood pressure associated with hypertension, and, in recent longer-term studies, ACE inhibitors (1) and AT1 receptor blockers (14, 35) have been shown to improve insulin insensitivity. In clinical trials using losartan or valsartan, there was a reduction in the risk of new-onset diabetes with an AT1 blocker (35), with similar results using the ACE inhibitor lisinopril (1). However, the precise mechanism by which RAS blockade improves insulin resistance and whether the benefits are due to central or peripheral effects are unknown. Nonetheless, with further study, RAS components may be an important therapeutic target for improved insulin resistance, and future
work will be required to establish whether effects of the brain RAS are involved.

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