Long-term systemic angiotensin II type 1 receptor blockade regulates mRNA expression of dorsomedial medulla renin-angiotensin system components

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Gilliam-Davis S, Gallagher PE, Payne VS, Kasper SO, Tommasi EN, Westwood BM, Robbins ME, Chappell MC, Diz DI. Long-term systemic angiotensin II type 1 receptor blockade regulates mRNA expression of dorsomedial medulla renin-angiotensin system components. Physiol Genomics 43: 829–835, 2011. First published May 3, 2011; doi:10.1152/physigendis.00167.2010.—In Fischer 344 (F344) rats, renin-angiotensin system (RAS) blockade for 1 yr with the angiotensin II type 1 (AT1) receptor blocker L-158,809 prevents age-related impairments in metabolic function, similar to transgenic rats with low glial angiotensinogen (Aogen). Brain RAS regulation may contribute to the benefits of long-term systemic AT1 antagonism. We assessed the mRNA of RAS components in the dorsomedial medulla of F344 rats at 3 (young; n = 8) or 15 mo of age (old; n = 7) and in rats treated from 3 to 15 mo of age with 20 mg/l of the AT1 receptor antagonist L-158,809 (Old+L; n = 6). Aogen and renin mRNA were lower in the young compared with old group. Angiotensin-converting enzyme (ACE) mRNA was lower in the old and Old+L compared with the young group. ACE2 and nephrilysin expression were significantly higher in Old+L compared with young or old rats. AT1b, AT2, and Mas receptor mRNA were higher with treatment. Leptin receptor mRNA was lower in the old rats and this was prevented by L-158,809 treatment. Dual-specificity phosphatase 1 (DUSP1) mRNA was highest in the Old+L group. Aggregate correlate summation revealed a positive relationship for Mas receptor mRNA with food intake. The findings provide evidence for regulation of dorsomedial medullary renin and Aogen mRNA during aging. Long-term AT1 receptor blockade increases the mRNA of the enzymes ACE2 and nephrilysin and the MAS receptor, which could potentially shift the balance from ANG II to ANG-(1–7) and prevent age-related declines in the leptin receptor and its signaling pathway. 

angiotensinogen; Fischer 344 rats; leptin; aggregate correlate summation

SYSTEMIC BLOCKADE of the renin-angiotensin system (RAS) with angiotensin II type 1 (AT1) receptor blockers or angiotensin-converting enzyme (ACE) inhibitors provides beneficial effects outside of the classic hemodynamic actions. RAS blockade improves many components of aging and reduces the onset of the metabolic syndrome and Type 2 diabetes (1, 8, 17, 43). However, the precise mechanisms underlying the beneficial effects are not entirely known. During aging, there is a decline in the circulating (systemic) RAS, including angiotensin (ANG)-(1–7) (30), suggesting that the beneficial actions of RAS blockade are partly due to the suppression of ANG II in tissues. Many tissues and organs contain their own local RAS, including the kidney, heart, vessels, adrenal gland, pancreas, and brain (4, 25). Increased brain ANG II activates sympathetic outflow, inhibits the baroreflex, stimulates thirst, and contributes to neurogenic hypertension (25, 36, 38). In rats with chronic renal failure, components of the brain RAS are upregulated, resulting in sympathetic overactivity and hypertension (29). Transgenic mice with increased production of brain ANG II developed hypertension and an increase in salt appetite and drinking volume (27, 28). In contrast, transgenic rats with a deficit in brain angiotensinogen (Aogen) (ASrAogen) do not develop hypertension, have reduced sympathetic nervous system activity and lower levels of glucose, insulin, and leptin during aging compared with age-matched Sprague-Dawley (SD) rats (20). ASrAogen rats also maintain baroreflex and insulin sensitivity associated with preservation of ANG-(1–7) over the course of aging compared with age-matched SD and (mRen2)27 rats (2, 21), suggesting a role for glial-produced ANG II in the metabolic impairments that occur during aging. In Fischer 344 (F344) rats, RAS blockade for 1 yr with the AT1 receptor antagonist L-158,809 prevented age-related impairments in metabolic function (decreased levels of insulin, glucose, leptin, and decreased body weight) relative to untreated age-matched rats (16). The treated F344 rats exhibit a metabolic profile similar to 15 to 18 mo old ASrAogen rats, implying that blockade of an age-induced activation of the endogenous brain ANG II may contribute to the benefits of long-term systemic AT1 antagonism. Few studies have documented age-related changes in the brain RAS components associated with ANG II vs. ANG-(1–7) generation. Therefore, our objective was to assess the gene expression of RAS components in the dorsomedial medulla of the F344 rats that were previously characterized for metabolic function and blood pressure to offer insight on the role of the brain RAS in the changes that occur during aging and the effects of long-term AT1 receptor blockade. In addition, a data-mining technique (6, 42) was used to assess potential relationships among variables studied in the F344 rats.

METHODS

Animals. All animals were obtained from Harlan Laboratories (Indianapolis, IN), and protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Three groups of male F344 rats were used in these studies: a young group (n = 8) at 3 mo of age, an old group (n = 7) at 15 mo of age, and a group treated with 20 mg/l of the AT1 receptor antagonist L-158,809 (n = 6) in their drinking water from 3 to 15 mo of age (1 yr).

Quantification of mRNA. Total RNA was isolated from the dorsomedial medulla (1 mm caudal to 3 mm rostral to the calamus scriptorius and above the level of the central canal at the caudal end to 2 mm below the floor of the fourth ventricle at the rostral end) of the three groups of rats using TRIZol reagent followed by incubation with
AMV reverse transcriptase in a mixture containing deoxynucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer. For real-time PCR amplification of the cDNA, gene-specific primers, TaqMan probes, and master mix were purchased from Applied Biosystems, and the reactions were performed on an ABI 7000 Sequence Detection System. 18S ribosomal RNA, amplified using TaqMan Ribosomal RNA Control Kit (Applied Biosystems), was used as the internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product is first detected, and defined as relative gene expression (ratio of target/control).

Statistical and data analysis. All data are presented as means ± SE, and P values < 0.05 were considered significant. One-way ANOVA with Tukey’s multiple-comparison post hoc tests was performed using Graphpad Prism.

Complex data relationships were assessed using correlate summation analysis (CSA). CSA illuminates the most covariant variables with respect to clustering, providing an alternative data-mining method comparable to PCA, without the need for specialized computer packages (6, 14, 42). A freely available Excel template that performs CSA for up to 100 variables for 4 groups of 15 subjects is available (http://en.wikipedia.org/wiki/Correlate_summation_analysis).

The aggregate correlate summation (ACS) utilized for these data included hemodynamic, renal, and metabolic variables previously obtained in these animals (16). Aging in the presence and absence of an AT1 receptor antagonist as a trajectory window of the RAS’s effects on disease development and degree of variable relationships were compared relative to normalized spread in the data (as a clustering surrogate). Variables were sorted using ACS rank for a layered correlation matrix/dimensioned (dd) group-wise descriptive statistics plot, to assist in analyzing the data (dd - data reduced by the mean of all subjects autonomous of group, for each variable).

RESULTS

Aogen mRNA was significantly higher in the older animals (P < 0.01) with a similar trend for renin mRNA. There was a further increase in the Old+L,158,809 group for both Aogen and renin mRNA (Fig. 1). ACE mRNA was lower in the old compared with the young, and this was partially reversed by treatment with the AT1 receptor antagonist (Fig. 2). Neither ACE2 mRNA nor nephrilysin mRNA was different between the old compared with the young animals; however, significantly higher values were observed in the Old+L,158,809 group compared with the young and old groups (P < 0.001). No differences were observed in the AT1a, AT1b, AT2, or Mas receptors mRNA between the old and young animals (Fig. 3). AT1b, AT2, and Mas receptor gene expression were twofold higher in Old+L,158,809 rats compared with young and old F344 rats, but AT1a receptor mRNA was not different among the groups (Fig. 3). Leptin receptor mRNA was significantly lower in the old rats compared with the young and L-158,809 treatment reversed this age-related change (Fig. 4). The mRNA expression of the p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3K) was significantly higher in the old and Old+L,158,809 groups compared with the young (Fig. 4). Dual-specificity phosphatase 1 (DUSP1) mRNA was higher in the Old+L,158,809 group compared with the young and old groups. There was no difference in the expression of protein tyrosine phosphatase nonreceptor type 1 (PTPN1) mRNA among the groups (Fig. 5).

Figure 6 shows the regression map from the correlate summation analysis of the data from the three groups of F344 rats. Using this visualization technique illustrates the concept behind ACS by grouping correlations to the upper left quadrant, while mean clustering spread diminishes to unity (left to right). The figure expresses both the clustering of the data and the correlations identified in this analysis. Aggregate correlate summation analysis showed a number of expected positive relationships between serum concentrations of insulin and glucose and serum leptin and body weight. In addition, the data reveal relationships between indices of metabolism and components of the RAS, including a positive relationship for Mas receptor mRNA with food intake and body weight and Aogen mRNA in the dorsomedial medulla with food intake and body weight, a negative relationship for the AT1a mRNA with food intake, and a positive relationship for p85 mRNA and body weight. Other correlations include a positive relationship for ACE mRNA in the dorsomedial medulla and systolic blood pressure (SBP) and a negative relationship between renin mRNA in the dorsomedial medulla with SBP. Interestingly, plasma ANG II concentration was a major predictor of the variance in the mRNA levels of AT1b, AT2, Mas receptor, and ACE2, as well as the PI3Kr1 (p85) mRNA in the dorsomedial medulla. DUSP1 mRNA had positive relationships with several RAS components including plasma ANG II and the dorsomedial medulla mRNA expression of Aogen, renin, nephrilysin, ACE2, and the Mas receptor. The summation analysis revealed positive relationships for PTPN1 mRNA with insulin, water intake, and the mRNA expression of Aogen, p85, and the Mas, AT2, and AT1b receptors.

DISCUSSION

In the older F344 rats, dorsomedial medulla Aogen mRNA was higher compared with the young animals, with a similar trend for renin mRNA. With respect to AT1 receptor antago-

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**Fig. 1.** Angiotensinogen (Aogen) mRNA was lower in the young (3 mo old) group vs. the old (15 mo old) and Old+L groups. Renin mRNA was significantly lower in the Young group compared with the Old+L group with a similar trend compared with the Old group. *P < 0.01 vs. Old; @P < 0.001 vs. Young and Old; #P < 0.05 vs. Young.
nism, Aogen and renin mRNA were significantly increased in the old group treated with the AT₁ antagonist compared with the old control group. One mechanism for the observed changes is blockade of the feedback inhibition on renin and Aogen by ANG II. Whether this is the mechanism for the changes evident in the brain requires further study, although the negative relationship identified between renin mRNA in the dorsomedial medulla and SBP further supports this concept. ACE2 and neprilysin mRNAs were significantly higher in the old treated group compared with the old group, while ACE mRNA declined with age but was restored with the AT₁ receptor blockade. We previously showed that ACE2 is the major ANG-(1–7) processing enzyme in dorsomedial medulla (9). There was no difference in AT₁a receptor mRNA expression between the groups, but AT₁b mRNA was significantly increased in the old treated group compared with the old group, while ACE mRNA declined with age but was restored with the AT₁ receptor blockade. We previously showed that ACE2 is the major ANG-(1–7) processing enzyme in dorsomedial medulla (9). There was no difference in AT₁a receptor mRNA expression between the groups, but AT₁b mRNA was significantly increased in the old treated group. AT₂ and Mas receptor mRNA levels were twofold higher in Old+L-158,809 rats compared with the old rats. The mRNA expression of the leptin receptor and the PI3K regulatory subunit p85 was significantly higher in the old treated rats vs. the old rats. DUSP1 mRNA expression was higher in the Old+L-158,809 group compared with the young and old groups. PTPN1 mRNA was similar among the groups. These findings provide evidence for increases in brain renin and Aogen mRNA expression in dorsomedial medulla during aging, and that long-term AT₁ receptor blockade favors the mRNA expression of enzymes (ACE2, neprilysin) and receptor (Mas) that would shift the balance from ANG II to ANG-(1–7) in this brain region. Importantly, the prevention of age-related declines in the leptin receptor mRNA expression and enhancement of mRNA expression of a leptin and insulin signaling pathway component (p85) provides putative mechanisms for preservation of metabolic function previously reported in treated F344 rats (16).

Hypertension and other disease states such as chronic renal failure are thought to be accompanied by an overactive brain RAS (29, 33). In young adult rats with aortic coarctation-induced hypertension, there were marked increments in Aogen and AT₁a receptor mRNA expression in brainstem areas controlling cardiovascular function and losartan treatment blocked the increase in AT₁a receptor mRNA expression. In addition, male Wistar rats treated with losartan had lower levels of brain Aogen and AT₁a receptor mRNA compared with controls (33). In the spontaneously hypertensive rat, candesartan treatment significantly increased brain Aogen, ACE, and AT₂ receptor mRNAs as well as AT₂ receptor protein. The Wistar-Kyoto rats treated with candesartan had significantly decreased Aogen and increased ACE mRNA in their brain microvessels compared with the vehicle controls (44). The increase in Aogen, ACE, and AT₂ receptor mRNAs from that study are similar to the results found in the normotensive rats in the present study. In general, long-term ANG II receptor blockade increases ANG II in plasma due to the loss of the feedback inhibition of renin release from the kidney. It was interesting that in our study, the plasma ANG II levels showed strong positive correlation with the mRNA for the AT₁b, AT₂, and Mas receptors and ACE2 in the dorsomedial medulla, without a major influence of the AT₁a receptor mRNA. With the AT₁ receptor occupied, elevated ANG II would theoretically bind the AT₂ receptor, which may be one possible mechanism that leads to beneficial effects of ANG II receptor blockade (7, 26). In addition, the levels of ACE2 mRNA in the present study were twofold higher than ACE mRNA, which suggest that the
system shifts from ANG II in favor of ANG-(1–7) production in the dorsomedial medulla during AT₁ receptor blockade. ANG-(1–7) has beneficial effects on vagal baroreflex function in this brain area and may contribute to the improvement in overall autonomic function. Further studies to determine whether the alterations in mRNA expression are reflected in actual changes in receptor or enzyme protein levels or binding and activity are warranted as protein levels may or may not parallel the changes in mRNA expression (44).

Hyperactivity of the brain RAS components favoring ANG II production may also contribute to insulin resistance since previous data revealed that rats with low brain Aogen do not develop insulin resistance during aging unlike their control counterparts (21). F344 rats, though normotensive during the aging process, develop insulin resistance, and animals treated with an AT₁ receptor blocker have lower serum insulin, glucose, and leptin levels compared with older control animals (16). While there are known interactions of ANG II and insulin in skeletal muscle (18, 35), the effects of AT₁ receptor blockade on the brain RAS cannot be ruled out as a contributing mechanism. Insulin and leptin both have actions at brain sites (receptors in paraventricular and arcuate nucleus and dorsal vagal complex) leading to impairment in baroreflex sensitivity and increased sympathetic nervous system activity and cross talk occurs among insulin, leptin, and ANG II receptors and signaling pathways (5, 39, 40). For example, insulin, leptin, and ANG II act at the PI3K pathway and the p85 regulatory subunit is an important component of the pathway. Impaired

Fig. 3. Angiotensin II type 1a (AT₁a) receptor mRNA did not differ among groups and the AT₁b receptor mRNA was significantly higher in the Old group compared with the Young and Old groups. AT₂ and Mas receptor mRNAs were significantly higher in the Old group compared with the Young and Old groups. *P < 0.01 vs. Young and Old; #P < 0.001 vs. Young.

Fig. 4. Leptin receptor mRNA was lower in Old rats, and this was prevented in the Old/H11001 rats. The p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3K) was significantly higher in both Old and Old/H11001 groups. *P < 0.01 vs. Old; #P < 0.05 vs. Old + L-158,809; @P < 0.001 vs. others.
activation of the PI3K pathway also occurs in insulin resistance and ANG II activity may play a role (32, 40). During aging, insulin and leptin sensitivity decline; however, this does not occur in the aging ASrAogen rats with low glial-derived Aogen. Our results suggest that prevention of age-related decline in the leptin receptor mRNA and the increased mRNA expression of the p85 regulatory subunit of PI3K provide potential mechanisms for preservation of metabolic function in the older L-158,809-treated F344 rats.

In addition to the PI3K and p85 signaling components, we investigated the mRNA expression of DUSP1 and PTPN1. DUSP1 inactivates mitogen-activated protein kinases (MAPKs) by dephosphorylation of the threonine and tyrosine residues (11, 24, 31). MAPKs are important in the pathogenesis of diabetes and are mediators of various vasoactive substances and growth factors, including ANG II (3). The negative feedback function of DUSP1 helps to regulate the signal transduction integrity of the MAPKs and, subsequently, various physiological and pathological processes. DUSP1 is decreased in the myocardium (41) and glomeruli (3) of rats made diabetic with streptozotocin. Rat mesangial cells exposed to high glucose had decreased DUSP1 (3). In rat vascular smooth muscle cells, hyperglycemia inhibited insulin-induced DUSP1 and PI3K expression (37). Wortmannin, the PI3K inhibitor, blocked insulin-
induced DUSP1 expression similar to hyperglycemic conditions suggesting that insulin regulates DUSP1 expression via the PI3K pathway (37). In streptozotocin-diabetic rats, the mRNA and the optical density and positive neuron number for DUSP1 were decreased in the CA1 and DG hippocampal area, which may play a role in diabetic dementia (45). We observed a higher expression of DUSP1 in the brain dorsomedial medulla of the Old+L-158,809 F344 rats compared with the young and old rats, and the expression was not different between the young and old groups. This suggests that ANG II blockade leads to increased mRNA expression of DUSP1 in the brain dorsomedial medulla of F344 rats, either directly or as a result of the various physiological responses associated with the blockade. Indeed, the shift in the balance of mRNA expression of receptors and enzymes favoring ANG-(1–7) may contribute to the increase in DUSP1, since ANG-(1–7) has been shown to activate MAPK phosphatases in vascular smooth muscle cells in culture (13). However, in rat neonatal cardiac myocytes, ANG II increased DUSP1 mRNA within 10 min, while the ANG II antagonist CV-11974 blunted the ANG II-induced increase in DUSP1 expression (19). ANG II increased DUSP1 gene expression within 15 min in adult rat ventricular myocytes and cardiac microvascular endothelial cells. The ANG II-induced activation of DUSP1 acutely in the ventricular myocytes was mediated by the AT2 receptor (12). Thus, the interactions between angiotensin peptides and DUSP1 mRNA may be time, tissue, and strain specific. PTPN1 dephosphorylates tyrosyl residues and is a potential target in treating Type 2 diabetes (22). PTPN1, which is expressed in all insulin-responsive tissues (23), negatively regulates insulin sensitivity and energy expenditure and promotes body fat accumulation (10, 23). PTPN1-deficient mice have increased insulin sensitivity, basal metabolic rate, total energy expenditure, and resistance to diet-induced obesity (10, 23). Of interest, we observed no difference in PTPN1 mRNA expression among the groups even though the young and Old+L-158,809-treated F344 rats had a better metabolic profile compared with the old rats (16).

The data-mining technique used (6, 14, 42) revealed several correlations for the variables previously determined (16) and the ones for this particular study as discussed above. The Mas receptor mRNA was positively correlated with food intake and body weight. Previous work revealed Mas receptor knockout in FVB/N mice caused a metabolic syndrome-like state including increased levels of insulin and leptin and insulin resistance and a 50% increase in abdominal fat mass (34). Moreover, chronic administration of ANG-(1–7) counteracts the metabolic syndrome (15). This suggests that the ANG-(1–7)/Mas axis is involved in the preservation of metabolic function as the correlation in this study implies.

We conclude that increases in brain renin and Aogen mRNA in dorsomedial medulla occur during aging in a normotensive rat model that develops insulin resistance and renal damage. Long-term AT1 receptor blockade promotes the mRNA expression of enzymes (ACE2, nephrilysin) and receptors (Mas) that could shift the balance from ANG II to ANG-(1–7) in this brain region. Moreover, the brain RAS may be involved in the impairments in metabolic function of the older rats since the expression of the leptin receptor and the PI3K subunit p85 were increased with long-term AT1 receptor blockade. Further studies assessing the protein levels and enzyme activities of brain RAS components are warranted since mRNA expression may not completely parallel functional changes in the system. However, several relationships between brain RAS components and indices of metabolic function were uncovered using a data-mining technique.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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