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Transgenic mice overexpressing human angiotensin I receptor gene are susceptible to stroke injury

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Abstract

Hypertension is one of the co-morbid conditions for stroke and profoundly increases its incidence. Angiotensin II (AngII) is shown to be at the center stage in driving the renin angiotensin system via activation of angiotensin 1 receptor (AT₁R). This makes the AT₁R gene one of the candidates whose differential regulation leads to the predisposition to disorders associated with hypertension. A haplotype block of four SNPs is represented primarily by haplotype-I, or Hap-I (TTAA), and haplotype-II, or Hap-II (AGCG), in the promoter of human AT₁R (hAT₁R) gene. To better understand the physiological role of these haplotypes, transgenic (TG) mice containing haplotype-I and II of the hAT₁R gene in a 166Kb BAC (bacterial artificial chromosome) were generated. Mice received injection of endothelin-1 (1mg/ml) directly in to the striatum and were evaluated for neurologic deficit scores and sacrificed for analysis of infarct volume and mRNA levels of various proteins. Mice containing Hap-I suffered from significantly higher neurological deficits (50%) and larger brain infarcts (60%) than Hap II. Similarly, the molecular analysis of oxidant and inflammatory markers in brains of mice showed a significant increase ($p < 0.05$) in NOX-1 (2.3 fold), CRP (4.3 fold) and IL6 (1.9 fold) and a corresponding reduced expression of antioxidants SOD (60%) and HO-1 (55%) in Hap-I mice as compared to Hap-II mice. These results suggest that increased expression of hAT₁R rendered Hap-I TG mice susceptible to stroke-related pathology, possibly due to increased level of brain inflammatory and oxidative markers and a suppressed antioxidant defense system.

Keywords

angiotensin receptor type 1; polymorphism; ischemic stroke; hypertension; endothelin 1

1 Introduction

Hypertension is considered the single most important risk factor for ischemic or hemorrhagic stroke [1]. Systemic or tissue specific Renin Angiotensin System (RAS) is the most studied mechanism in understanding blood pressure (BP) physiology. Although

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clinical studies have shown an association between hypertension and higher stroke risk [2], the role of RAS has not been studied fully.

The classical view of RAS is that angiotensinogen produced in the liver is cleaved to angiotensin I (AngI) by renin in circulation, followed by its conversion by angiotensin converting enzyme (ACE) to the vasoactive octapeptide, angiotensin II (AngII). A large number of studies have identified the existence of the systemic RAS components in the brain [3–6]. Besides the regulation of body water balance, BP maintenance, vasopressin release, and sexual behavior, brain RAS has also been implicated in the regulation of cerebral blood flow, cerebroprotection, stress, and memory consolidation (reviewed by John and Harding, 2013, [7] The effector peptide of the RAS, AngII, exerts the majority of its physiological effects via the G-protein coupled receptor, angiotensin 1 receptor (AT₁R). A large amount of evidence supports the contribution of AT₁R in cerebrovascular pathologies [8,9]. In addition, activation of AT₁R stimulates the central and peripheral sympathetic systems and increases cerebrovascular vasoconstriction [10,11]. These observations are supported by the findings of increased expression of endothelial AT₁R (a signal of increased AT₁R stimulation) in brain microvessels and the middle cerebral artery (MCA) of spontaneously (genetic) hypertensive rats (SHRs) when compared to the normotensive controls [8]. Etiopathogenesis of atherosclerosis also involves vascular reactive oxygen species (ROS), which not only act as modulators of vascular tone but also as the second messenger to alter the vascular cell phenotypes. AngII induces production of ROS, which is one of the most important mediators of the atherogenic actions of RAS [12,13]. Studies of SHRs also show that increased AT₁R expression altered the expression of endothelial nitric oxide synthase (eNOS), tumor necrosis factor α (TNF- α), nuclear factor (NF- κ), interleukin 1 β (IL-1 β) and heat shock protein 70 (HSP70), causing increased macrophage infiltration in cerebral microvessels and, finally, cerebrovascular inflammation. The increased inflammation and decreased vascular compliance in the cerebrovasculature due to over-expression of AT₁R in SHRs were successfully reversed by AT₁R blocker, candesartan [14–16]. Favoring these studies, we have recently published our results showing hypertension in TG mice overexpressing the human AT₁R (hAT₁R) gene [17]. Recent studies also show a reduction in age-related development of hypertension in AT₁R deficient mice [18]. Overall, these studies indicate that increased AT₁R gene expression may contribute to the onset of hypertension and other associated tissue-specific anomalies.

Since polymorphisms in the promoter region can affect the expression of a gene by transcriptionally mediated mechanisms, we have examined the potential role of single nucleotide polymorphisms (SNPs) in the 5'-flanking region of the hAT₁R gene in hypertension. We have found that variants –810T, –713T, –214A, and –153A always occur together [named haplotype-I (Hap-I), representing SNPs TTAA] and variants –810A, –713G, –214C, and –153G always occur together [named haplotype-II, (Hap-II) representing SNPs AGCG] (Fig. 1). We have shown that Hap-I of the hAT₁R gene is associated with hypertension in the Caucasian population and has increased promoter activity on transient transfection of reporter constructs [17]. To better understand the molecular regulation and physiological roles of these haplotypes, we have generated transgenic (TG) mice containing Hap-I and II of the hAT₁R gene in a 166 Kb bacterial artificial chromosome (BAC). We observed that Hap-I TG mice have increased hAT₁R

mRNA levels and increased expression of inflammatory markers, oxidative stress and BP, as compared to hAT₁R TG mice containing Hap-II [17].

In the present study, we hypothesized that Hap-I TG mice subjected to endothelin-1 (ET-1) induced focal ischemic injury will suffer from higher neurologic deficits (NDS) and larger infarct volumes compared to the Hap-II TG mice or C57 control mice due to increased brain inflammatory and oxidative markers and suppressed antioxidant defense systems.

Methods

Generation of TG mice

All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional IACUC committee at The University of Toledo. The TG mice utilized in this study were generated in our lab, as previously described by Jain et al. [17]. Genotyping analysis of the tail snips, followed by sequencing, was performed to confirm the genetic lineage of these TG mice. As previously described, the TG mice with Hap-I (TTAA) have variants –810T, –713T, –214A, and –153A, whereas TG mice with Hap-II have variants –810A, –713G, –214C, and –153G (Fig. 1). Initially, we obtained three founder lines from each construct, but after characterization, we maintained one line from each TG construct. These TG mice have a single copy of the hAT₁R gene, as determined by quantitative-PCR [19]. Male TG animals were backcrossed with female C57 mice for seven generations, and all future experiments were performed using the heterozygous animals. All animals were randomized and distributed into different groups. We used three- to five-month-old male mice, 9 mice in each group for studying the neurologic deficit scores and the infarct volume analysis, and 6 mice in each group for quantitative real time PCR studies.

Endothelin-1 induced focal brain injury

Focal ischemia was induced in 3–5 months old, male C57, Hap-I and Hap-II mice by giving a ET-1 intra striatal injection using the method of Soyulu et al. [20]. Mice were anesthetized for a brief period in 4–5% of isoflurane in the induction chamber and then stabilized at 1–2% isoflurane using the nose cone on the stereotaxic equipment (Stoelting, Wood Dale, IL). Core body temperature was maintained between 37–37.5°C using a rectal thermometer and heating blanket throughout the surgery. Aseptic conditions were maintained by using sterile drapes, gloves and autoclaved surgical equipment. A thin midline incision was made to the scalp, and a small burr hole was made on the right hemisphere of the skull. Using a 5µl Hamilton syringe, vasoconstrictor endothelin-1 (ET-1) (1mg/ml dissolved in 0.9% saline; Sigma-Aldrich, St. Louis, MO, USA) or 0.9% saline was injected using the following coordinates: 1.0mm anterior, and 1.0mm lateral, to bregma and 1.2mm below the pia. A total volume of 1µl was injected at the infusion rate of 0.1µl/min, and the needle was slowly withdrawn to prevent the backflow of ET-1 or saline. The hole was covered with bone wax and the incision was sutured. After surgery, mice were placed in a temperature-controlled incubator for 2h and shifted to home cages after complete recovery from surgery.

Neurologic deficit scores

Neurological deficits induced by ET-1 injection were evaluated on a 28-point scoring pattern optimized by our group [21]. A person unaware of the experimental design, but an expert in the evaluation of the NDS, evaluated NDS 72h after ischemia; evaluation included both sensory and motor deficits such as body symmetry, gait, climbing, compulsory circling, front limb symmetry, bench top circling and whisker response. Each of the seven tests was graded from 0 to 4, with higher scores indicating severe deficits.

Infarct volume analysis

All animals were euthanized at 72h after ET-1 induced ischemia. Brains were dissected and sliced into five 2-mm thick coronal sections and incubated in 1% triphenyltetrazolium chloride (TTC, Sigma Co. MI). Infarct area was analyzed from five sections of each brain, the rostral and caudal side of each slice in combination with thickness, and expressed as percentage of damage on the contralateral hemisphere. An investigator to whom the treatment method was unknown measured the infarct volume with the help of ImageJ software provided by NIH.

Quantitative real-time (qRT) PCR

The brains were harvested and snap-frozen in liquid nitrogen. The isolated tissues were stored at -80°C until utilized for further experiments. RNA was extracted from the borders of the ischemic stroke using the RNeasy Plus mini kit (Qiagen, Valencia, CA). We targeted the similar size and region of the ischemic brain from all the groups of mice to keep the uniformity in result comparisons. One microgram of RNA was reverse-transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Pittsburgh, PA), as described in the manufacturer's protocol. QRT-PCR was performed using primers for human AT_1R , mice AT_1R , IL6, CRP, NOX-1, SOD-1 HO-1 and GAPDH genes that were obtained from Super Array Bioscience Corporation (Frederick, MD) or from Integrated DNA Technologies (Coralville, IA). Gene expression was examined using the Power SYBR green master mix on the ABI 7500 Fast Real-Time PCR system from Life Technologies (Carlsbad, CA). Following a 95°C incubation for 10min, 40 cycles of PCR ($95^{\circ}\text{C}/30\text{s}$; $60^{\circ}\text{C}/30\text{s}$), were then performed using $1\mu\text{l}$ of cDNA, 50nM PCR primers and $12.5\mu\text{l}$ SYBR Green PCR Master Mix in $25\mu\text{l}$ reactions. Threshold cycles for three replicate reactions were determined using MxPro-Mx3005P software (version 4.10), and relative transcript abundance was calculated following normalization with mouse GAPDH. In general, relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as untreated control. This is a widely used method to present relative gene expression by comparative C_T method, also referred to as the 2^{-C_T} method [22].

Statistical analysis

One-way ANOVA, followed by a Newman-Keuls multiple comparison test, was used to compare sham and the different treatment groups. In cases where the data was not normally distributed or the variance was not homogeneous, non-parametric tests were used. $p < 0.05$ was considered statistically significant for all tests.

Results

TG mice with haplotype-I show a higher neurologic deficit and a larger infarct volume 72h after striatal endothelin-1 injection

C57, Hap-I and Hap-II mice were tested for neurologic deficit scores (NDS) the day before injection and after 72h. Mice were sacrificed for infarct volume analysis by 1% triphenyltetrazolium chloride (TTC) staining at 72h. TG mice containing Hap-I suffered from 50% higher neurologic deficits (Fig. 2B) and 60% larger infarct volume (Fig. 2C) as compared to the Hap-II TG or C57 control mice (* $p<0.05$ vs. Hap-II and C57; $^{\dagger}p<0.05$ vs. Hap-I and C57; $n=9$ each group). The results of this study support our hypothesis that Hap-I has a role in stroke pathophysiology.

Expression of human AT₁R is increased in brains of TG mice with haplotype-I 72h after endothelin 1 injection

To elucidate the impact of variable hAT₁R haplotypes on stroke injury induced by ET-1 injection, we quantified the expression of hAT₁R post injury and compared it with the basal levels (sham). We observed that hAT₁R increases by more than fivefold in mice expressing Hap-I as compared to less than twofold change in mice containing Hap-II (* $p<0.05$ vs. sham in the same group; $^{\dagger}p<0.05$ vs. stroke in Hap-II; * $p<0.05$ vs. Hap-II sham) (Fig. 3A). Interestingly, the mouse AT₁R levels changed significantly in mice with Hap-II and C57 controls (Fig. 3B), indicating a major role of human AT₁R in stroke pathogenesis in mice with Hap-I of hAT₁R. These results also suggest the role of various polymorphic sites in two haplotypes of hAT₁R contributing towards their differential expression at basal levels and after stroke.

Expression of the pro-inflammatory and oxidative markers is increased in the brains of TG mice containing haplotype-I of the hAT₁R gene

To assess the functional relevance of the up-regulated hAT₁R, expression analysis of the pro-inflammatory and oxidative markers, including interleukin 6 (IL6), C-reactive protein (CRP), and NADPH-oxidase, was performed. Complementary experiments, using hemeoxygenase (HO1) and superoxide dismutase-1 (SOD-1), were performed to examine the cellular antioxidant defenses. At basal levels (without ischemia), Hap-I TG mice were observed to have (a) significantly higher expression of proinflammatory markers including IL6 (Fig. 4A), (b) significantly higher expression of oxidative markers, CRP and NOX-1 (Fig. 4B–C), and (c) reduced levels of antioxidant HO-1 and SOD (Fig. 5A–B) compared to the Hap-II or C57 control mice. Interestingly, all these parameters were observed to worsen further (IL6, 1.9 -fold; CRP, 4.3 -fold; NOX-1, 2.3 -fold) once Hap-I TG mice were subjected to the ET-1 injection and brain tissues were collected for mRNA analysis after 72h ($p<0.05$). Combined results from figures 3, 4 and 5 suggest that basal or stroke-induced increases in hAT₁R expression in Hap-I TG mice promote a proinflammatory/oxidant milieu, along with a decrease in antioxidant potential, as compared to the TG mice containing Hap II or C57 non-TG controls.

Discussion

In the present study, we showed that mice containing Hap-I suffered from significantly higher neurological deficits and larger brain infarcts after receiving an injection of ET-1 directly into the striatum. The molecular analysis of oxidative stress and inflammatory markers in post-ischemic mouse striata (border of the ischemic area) showed a significant increase in NOX-1, CRP and IL6, along with a corresponding reduced expression of antioxidants SOD and HO1, in Hap-I mice as compared to Hap-II mice. These results suggest that increased expression of hAT₁R renders Hap-I TG mice susceptible to stroke-related pathology, possibly due to increased inflammation and insufficient antioxidant defense mechanism.

Chronic hypertension damages arteries throughout the body; in particular, the brain's microcirculation is at higher risk of narrowed or clogged arteries and weakened vessel walls. All these consequences increase the risk of ischemic and hemorrhagic stroke in the population with hypertension as a comorbid condition, evidenced by many clinical studies [1]. There is also overwhelming evidence that supports the notion that AngII is involved in atherosclerosis through the AT₁ receptor-mediated activation of pro-hypertensive, inflammatory, oxidative, and metabolic effects. AT₁R is the principal mediator of the majority of the physiological effects of AngII, and mounting evidence shows the contribution of AT₁R in cerebrovascular pathologies [8,9].

Diseases like hypertension and stroke are polygenic in nature, with a strong familial inheritance. It is believed that single nucleotide polymorphisms (SNPs) in the regulatory regions of the genes regulating BP contribute to this inheritance, and the AT₁R gene is one of the prime candidates whose differential regulation leads to the predisposition to such disorders. In this perspective, the observed haplotype block of four SNPs, represented primarily by Hap-I (TTAA) and Hap-II (AGCG) in the promoter of the hAT₁R gene, provide a better model to understand the pathophysiology associated with it (Fig. 1) [17]. In our present study, we wanted to better understand the role of these haplotypes in the progression of diseases like stroke. We observed that mice containing these haplotypes suffered from higher neurologic deficits and infarction as compared to Hap-II or sham mice when subjected to ET-1 striatal injection-induced stroke. These observations are in consonance with the previous studies suggesting that heightened inflammation, oxidative stress and vascular dysfunction in hypertensive subjects leads to the increased risk of stroke.

We selected the ET-1 model of transient ischemia to assess the vascular dysfunction that is profoundly induced by ET-1 striatal injection and compare the pathophysiologies between different haplotypes. The brain pathology induced by ET-1 generates intense vasoconstriction, inflammation and free radical damage and, most importantly, it has been implicated to cause vascular dysfunction [23]. Importantly, we were not sure about the mortality rates in these haplotypes against the severe type, 90-min filament-occluded model of transient middle cerebral artery occlusion ischemia. Therefore, we sought to investigate the brain pathophysiology of these haplotypes in the less severe ET-1 striatal injection-induced ischemia model, which is specific for vascular dysfunction and induces inflammation and free radical damage.

Focal ischemia-induced neurovascular injury is accompanied by significant up-regulation of AT₁R. Interestingly, this induction of AT₁R is non-specific and is observed in both mouse and human genes. However, where the mouse gene only showed two- to fourfold induction, the human gene was induced by greater than 30-fold (Fig. 3). Of most significance is the finding that this AT₁ gene overexpression, by focal ischemia-induced neurovascular injury, is haplotype-dependent. Haplotype I exhibits up-regulation of the hAT₁R gene, not haplotype II; the mouse gene is induced in all models except haplotype I (Fig. 3). This is possibly due to the presence of stronger transcription factor binding sites in the promoter of Hap-I hAT₁R gene that compete for the existing pool of trans-activating proteins. This increases the transcriptional activity of hAT₁R in Hap-I mice as compared to the mAT₁R gene. This is further supported by an increase in mAT₁R expression in Hap-II TG mice that do not offer similar affinity to the transcription factors as the Hap-I transgene.

Activation of AT₁R up-regulates several inflammatory and oxidative markers, while suppressing certain antioxidant systems [24,25]. Hap-I transgenic mice that overexpress the hAT₁R gene demonstrate an inflammatory, oxidative milieu with impaired cellular antioxidant defenses. This AT₁R dependent, pro-oxidant, inflammatory setting underlies an enhanced post-stroke ischemic insult in these mice. A blunted, post-ischemic response in the Hap-II TG and C57 mice highlight the contribution of hAT₁R haplotype in bringing about this ischemic neuropathology. These findings implicate the imperative role of Hap-I AT₁R receptor in the pathophysiology of stroke.

Conclusions

In summary, our study for the first time showed that Hap-I AT₁R pathophysiology causes increased inflammation, oxidative stress and vascular dysfunction and makes mice more susceptible to stroke-induced injuries. This is the first study that has defined the role of Hap-I AT₁R polymorphism in stroke, and we believe this mouse model will open avenues to understand its role in other neurodegenerative diseases.

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Figure 1.

Schematic presentation of BAC plasmids containing hAT₁R gene represented by Hap-I and Hap-II. This BAC contains 68kb of the 5' flanking region, 45kb of the coding region and 53kb of the 3' flanking region of the hAT₁R gene cloned in 7.4kb of the pBeloBAC11 vector. Nucleotide sequence of the wild type BAC revealed that it contains haplotype-I of the hAT₁R gene. The 1.2Kb promoter of the hAT₁R gene in this BAC was modified to haplotype-II by re-combineering, using a 1.2Kb promoter fragment from a human subject containing haplotype-II of the hAT₁R gene. Variants -810T, -713T, -214A, and -153A

always occur together (named haplotype-I or Hap-I, representing SNPs TTAA) and variants –810A, –713G, –214C, and –153G always occur together (named haplotype-II or Hap-II, representing SNPs AGCG).

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Figure 2.

Hap-I mice show higher neurologic deficit and a larger infarct volume 72h after striatal endothelin-1 injection. C57, Hap-I and Hap-II mice were subjected to striatal ET-1 injection and tested for neurologic deficit scores (NDS) the day before injection and after 72h. Mice were sacrificed for infarct volume analysis at 72h. **A.** Representative picture showing the stereotaxic coordinates used to inject ET-1 into the mouse striatum. **B.** TG mice containing Hap-I suffered from higher neurologic deficits and a larger infarct volume as compared to the Hap-II TG or C57 control mice (* $p < 0.05$ vs. Hap-II and C57; † $p < 0.05$ vs. C57; $n = 9$ each group).

Figure 3.

Expression of human AT₁R is increased in brains of haplotype-I mice 72h after endothelin 1 injection. C57, Hap-I and Hap-II mice were subjected to striatal ET-1 injection, and brains were removed after 72h. **A.** Graphs showing mRNA levels of hAT₁R in brains of different TG mice at basal levels and after ischemic stroke. hAT₁R increases by more than fivefold in mice expressing Hap-I, as compared to less than twofold change in mice containing Hap-II. **B.** Graph showing mRNA levels of mouse AT₁R in brains of different TG mice at basal levels and after ischemic stroke. Mouse AT₁R levels differed significantly in mice with Hap-II and C57 controls. (* $p < 0.05$ sham vs. stroke in the same group; † $p < 0.05$ sham or stroke in Hap-I vs. sham or stroke in Hap-II or C57 controls. $n = 6$)

Figure 4.

Expression of the pro-inflammatory and oxidative markers is increased in the brains of TG mice containing Hap-I. C57, Hap-I and Hap-II mice were subjected to striatal ET-1 injection and brains were isolated after 72h. A–C. Graphs showing basal and post ischemic mRNA levels of pro-inflammatory/oxidative markers; interleukin 6 (IL6), C-reactive protein (CRP) and NADPH-oxidase in different groups. (* $p < 0.05$ sham vs. stroke in the same group; † $p < 0.05$ sham or stroke in Hap-I vs. sham and stroke in Hap-II and C57, $n = 6$)

Figure 5.

Antioxidants show a reduced basal and post ischemic expression in the brain of TG mice with Hap-I. Heme oxygenase (HO1) and superoxide dismutase-1 (SOD-1) show a reduced expression in Hap-I TG mice when compared to the C57 control mice or the mice containing Hap-II AT₁R. Their expression further declines, more significantly in Hap-I AT₁R mice, after ET-1 treatment. (* $p < 0.05$ sham vs. stroke in the same group; † $p < 0.05$ sham or stroke in Hap-I vs. sham and stroke in Hap-II and C57, $n=6$)