Spontaneously Hypertensive Rats Are Highly Vulnerable to AMPA-Induced Brain Lesions

Clotilde Lecrux, PhD; Olivier Nicole, PhD; Laurent Chazalviel, BSc; Christelle Catone, PhD; Julien Chuquet, PhD; Eric T. MacKenzie, PhD; Omar Touzani, PhD

Background and Purpose—Whereas the effects of chronic arterial hypertension on the cerebral vasculature have been widely studied, its effects on brain tissue have been studied less so. Here we examined if spontaneously hypertensive rats (SHRs) or the normotensive control Wistar Kyoto rats (WKYs) made hypertensive by renal artery stenosis (R-WKYs) are vulnerable to an excitotoxic brain lesion provoked by an overactivation of glutamate receptors.

Methods—Lesion volumes were quantified by histology in WKYs and SHRs subjected to striatal administration of N-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). The expression of AMPA receptors subunits and calcium/calmodulin kinase-IIα was analyzed by real-time polymerase chain reaction and Western blot.

Results—NMDA (50 and 75 nmol) induced similar lesions in both SHRs (10±2 mm³ and 16±4 mm³, respectively) and WKYs (11±2 mm³ and 19±7 mm³, respectively). However, AMPA-induced (2.5 and 5 nmol) lesions were significantly greater in 14-week-old SHRs (14±3 mm³ and 20±5 mm³, respectively) than WKYs (4±2 mm³, P<0.05 and 7±4 mm³, P<0.001, respectively). Furthermore, normotensive 7-week-old SHRs also displayed an aggravated AMPA-induced lesion compared with age-matched WKYs (10±3 mm³ vs 6±3 mm³; P<0.05). Neither NMDA nor AMPA produced increased lesion volumes in R-WKYs (12±3 mm³ and 5±4 mm³, respectively) compared with WKYs. Striatal levels of AMPA receptors subunits, GluR1 and GluR2, were not different between SHRs and WKYs. However, SHRs displayed an increase in phosphorylated form of GluR1 at Ser-831 (P<0.001 vs vehicle).

Conclusions—These findings show that an increase in phosphorylated GluR1, which increases AMPA receptor conductance, may be involved in the vulnerability of SHRs to AMPA. (Stroke. 2007;38:3007-3015.)

Key Words: AMPA receptor □ arterial hypertension □ CaMKII □ cerebral ischemia □ excitotoxicity □ GluR1

Animal models of chronic arterial hypertension, especially those of spontaneously hypertensive rats (SHRs), have been commonly used to investigate the effect of chronic arterial hypertension as an aggravating factor for stroke.¹ Functional and structural vascular alterations caused by chronic arterial hypertension are typically put forward to explain the hypertension-related increase in ischemic brain damage.² Such alterations would aggravate cerebral blood flow (CBF) after arterial occlusion. However, some data exist to suggest that in SHRs, neurons and glial cells may exhibit an intrinsic vulnerability, which may participate in the exacerbation of brain damage after a cerebral insult.³⁻⁶

In the present study, we aimed to assess if there is a vulnerability of brain tissue in SHRs that might contribute to increase brain damage after cerebral ischemia. As a first step, we compared the evolution of CBF during transient ischemia, as well as the resulting infarction in SHRs and Wistar Kyoto rats (WKYs). We then particularly directed our experimental paradigm toward glutamate, the major excitatory transmitter in the brain. This neurotransmitter plays a critical role in the cellular events after ischemic stroke. After an ischemic insult, the increased release of glutamate results in an overactivation of ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors that creates an excessive calcium influx, which in turn triggers deleterious intracellular processes leading to neuronal death.⁷ Accordingly, we examined the magnitude of brain damage induced by the administration of either NMDA or AMPA into the striatum of hypertensive and normotensive rats. Because hypertension gradually develops in SHRs, we used 7-, 14-, and 25-week-old SHRs. These ages were selected in AMPA studies because 7-week-old SHRs display...
moderately elevated arterial pressure, and hypertension is fully established in 14- and 25-week-old SHRs.8 We also used an alternative model of WKY rats in which arterial hypertension was induced by renal artery stenosis (R-WKYs). This model in which arterial hypertension is acquired represents a control group, the value of which was to estimate the impact of the specific genetic background of SHRs on their susceptibility to excitotoxic insults. Finally, we analyzed whether the increased sensitivity of SHRs to AMPA administration is linked to a differential expression of AMPA receptors subunits in the striatum. GluR1 and GluR2 are the principal subunits of AMPA receptors involved in neuronal death.9 Furthermore, several forms of regulation of AMPA receptors have been identified. Of particular interest is a phosphorylation site at Ser831 in the C-terminal tail of the GluR1 subunit that plays a central role in channel localization and biophysical properties.10,11 The major kinase responsible for selective phosphorylation of Ser831 in GluR1 is the Ca2+/calmodulin kinase (CaMKII), and previous data have demonstrated that the level of CaMKII can be modified in the myocardium of SHRs.12 Here, we postulate that the α form of CaMKII (CaMKIIα) could be involved in the increase of pGluR1 in SHRs.

Materials and Methods

Animals

All these experiments were performed on male WKY and SHR rats purchased from R. Janvier Breeding Center; Le Genest St. Isle, France. When indicated, animals were used at 3 ages: 7, 14, and 25 weeks old. This study was conducted in accordance with the appropriate European Directives and French National Legislation. All the surgical procedures were performed randomly on the animals of each strain at every age. Data analyses (including lesion volume, CBF values, and protein expression) were performed by investigators blind to the strain of the animal. Animals that did not survive within the defined time point were excluded.

Anesthesia and General Preparation

Anesthesia was performed with sevoflurane in an O2:N2O mixture (30%:70%). Related to the potential pain caused by the surgical approach, higher levels of sevoflurane were used during the ischemia (3.5% to 4.0%), compared with the intrastriatal injection approach, during the ischemia (30%:70%). Related to the potential pain caused by the surgical approach, higher levels of sevoflurane were used during the ischemia (3.5% to 4.0%), compared with the intrastriatal injection approach, during which only 2.5% sevoflurane was used. After the end of the injection, the needle was then left in place 10 minutes before withdrawal. To investigate the effects of CaMKIIa blockade on AMPA-induced brain damage, a group of rats received an intrastriatal injection of an inhibitor of this kinase (KN-93, 0.4 mmol/L; Sigma) 30 minutes before the administration of AMPA. The animals were euthanized 48 hours later for histological analyses because previous studies using AMPA or NMDA indicated that a well-developed lesion volume is obtained at this time point.

Measurement of Lesion Volume

After deep anesthesia, rats were euthanized by decapitation and the brains were removed rapidly and frozen in isopentane at −65°C. Whole brains were cut in 20-μm-thick sections with a cryostat (Leica). The sections were collected on glass slides and stained with thionin. The lesioned area was quantified by image analysis (Scion Image). Infarction volume was corrected for edema as described previously.

Two-Kidney, One-Clip Renovascular Hypertension

Under sevoflurane anesthesia (4.5%), 5-week-old WKYs were subjected to an abdominal incision and a U-shape silver clip (0.2 mm) was placed around the left renal artery to induce a stenosis. The level of arterial pressure was tested weekly by the tail-cuff method in these R-WKYs.16 (LE5002; Letica). These rats were subjected to excitotoxic lesions 9 weeks after the surgery.

Western Blot Analysis

Striata were dissected from WKYs and SHRs (14 weeks old, n=3). All the protocol of extraction was performed on ice to reduce all enzymatic activities. Moreover, the lysis buffer has been supplemented with proteases inhibitors cocktail (Sigma-Aldrich) and phosphatase inhibitors cocktail (Sigma-Aldrich). After isolation, small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid protein assay method (Pierce, Rockford, Ill) using bovine serum albumin as a standard. Equal amounts of protein (20 μg for brain extracts) were loaded onto 8% acrylamide gels. The proteins were separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes (0.2 μm). Primary antibodies used include a rabbit antibody for GluR1 (1:200; Chemicon), GluR2 (1:200; Chemicon) or phosphoGluR1 (pGluR1) at Ser831 (1:1000; Upstate Biotechnology) and revealed with a anti rabbit IgG peroxidase-conjugated (1:30 000; Sigma-Aldrich) or for CaMKII (1:200; Santa Cruz) revealed with a anti-mouse peroxydase-conjugated (1:200; Chemicon) or phosphoGluR1 (pGluR1) at Ser831 (1:1000; Upstate Biotechnology) and revealed with a anti rabbit IgG peroxidase-conjugated (1:30 000; Sigma-Aldrich) or for CaMKIIa (1:200; Santa Cruz) revealed with a anti-mouse peroxydase-conjugated (1:1000; Sigma-Aldrich). The enhanced chemiluminescence immunoblotting detection system (Amersham). The amount of protein transferred onto the membrane was confirmed by immunoblotting for GAPDH (Chemicon). Chemiluminescence was detected by autoradiography. Several film exposures were obtained for each set of samples to insure that signals were within a linear range allowing an accurate quantification of the enhanced chemiluminescence signal. Bands were quantified by analysis of scanned images by ImageJ software (http://rsb.info.nih.gov/ijs).
Statistical Analysis

The results are presented as mean±SD. Statistical comparisons between 2 groups were analyzed by Student t tests. ANOVA and Fisher posttests were used for multiple comparisons. \( P < 0.05 \) was accepted as being indicative of a significant difference.

Results

Cerebral Ischemia-Induced Damage

Compared with age-matched WKYs, MCA occlusion of 90 minutes induced greater cerebral ischemic infarction in both 7-week-old and 14-week-old SHRs (Figure 1A). One SHR died prematurely and was subsequently excluded from the analysis. The decrease in CBF, estimated by laser Doppler flowmetry, during transient MCAO was similar in SHRs and WKYs at both ages (Figure 1B).

MAP before the induction of MCAO was higher in 14-week-old SHRs compared with age-matched WKYs (\( P < 0.05 \)), and compared with 7-week-old WKYs and SHRs (\( P < 0.01 \)). There was no significant difference in MAP between 7-week-old SHRs and age-matched WKYs. After the induction of ischemia, MAP increased in SHRs. Arterial pH, \( \text{PACO}_2 \), \( \text{PaO}_2 \), and rectal temperature were maintained within physiological limits in all 4 groups (Table).

Table. Physiological Parameters Before, During, and After Transient MCAO

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>MAP (mm Hg)</th>
<th>Before Occlusion</th>
<th>During Occlusion</th>
<th>15-min Reperfusion</th>
<th>( \text{PACO}_2 ), mm Hg</th>
<th>( \text{PaO}_2 ), mm Hg</th>
<th>pH</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>14</td>
<td>92.9±7*</td>
<td>91.1±13‡</td>
<td>94.0±23</td>
<td>36.9±3</td>
<td>165±47</td>
<td>7.43±0.03</td>
<td>37.5±0.3</td>
</tr>
<tr>
<td>SHR</td>
<td>14</td>
<td>108.2±9</td>
<td>120.5±21</td>
<td>118.8±29</td>
<td>36.4±5</td>
<td>197±37</td>
<td>7.45±0.05</td>
<td>37.6±0.3</td>
</tr>
<tr>
<td>WKY</td>
<td>7</td>
<td>85.2±11†</td>
<td>82.4±7‡</td>
<td>79.6±9</td>
<td>35.4±4</td>
<td>179±13</td>
<td>7.42±0.06</td>
<td>37.3±0.3</td>
</tr>
<tr>
<td>SHR</td>
<td>7</td>
<td>84.8±15†</td>
<td>94.7±8‡</td>
<td>89.9±9</td>
<td>35.4±3</td>
<td>183±9</td>
<td>7.48±0.04</td>
<td>37.5±0.3</td>
</tr>
</tbody>
</table>

For MAP: *\( P < 0.05 \), †\( P < 0.01 \), ‡\( P < 0.001 \) vs 4-week-old SHR; §\( P < 0.05 \) vs baseline. ANOVA followed by Fisher PLSD.

Other parameters are given as mean throughout the whole experiment under sevoflurane anesthesia and are maintained within physiological limits. All data are given as mean±SD.
NMADA-Induced Excitotoxic Damage

The volumes of NMADA-induced lesions were near identical in 14-week-old WKYs and SHRs either for a dose of 50 nmol (11±2 mm³ and 10±2 mm³, n=7 and 9, respectively; Figure 2A) or 75 nmol (19±7 mm³ and 16±4 mm³, n=8 and 7, respectively; Figure 2B). Throughout the whole period of these experiments, the level of MAP was significantly higher in SHRs compared with WKYs (P<0.01; Figure 2A and 2B). NMADA-lesion volume was not increased after induction of chronic hypertension in R-WKYS compared with normotensive WKYs (12±3 mm³ and 14±3 mm³, respectively, n=6 in each group; Figure 2C). The level of MAP was also significantly increased in R-WKYS compared with WKYs (P<0.01; Figure 2C).

We failed to observe any significant variation of MAP under anesthesia after the injection of NMADA in any of the groups analyzed. All the other physiological parameters recorded were similarly maintained within physiological ranges (data not shown).

AMPA-Induced Excitotoxic Damage

Intrastriatal injection of 5 nmol of AMPA provoked greater damage in 14-week-old SHRs compared with age-matched WKYs (20±5 mm³ and 7±4 mm³, n=3 and 5, respectively; P<0.05; Figure 3A) and was associated with higher mortality rate in SHRs (63%) relative to WKYs (17%). To reduce this AMPA-induced high rate of mortality in SHRs, we subsequently tested a lower dose of AMPA. Lesion volumes induced by 2.5 nmol of AMPA in 14-week-old SHRs were also considerably higher compared with age-matched WKYs (14±3 mm³ and 4±2 mm³, n=6 and 7, respectively; P<0.001; Figure 3B). Greater brain damage was also found in older SHRs (Figure 3B). Importantly, 7-week-old SHRs with mild hypertension displayed as well an exacerbated lesion relative to that found in 7-week-old WKYS (10±3 mm³ and 6±3 mm³, n=7 and 6, respectively; P<0.05; Figure 3B). With 2.5 nmol of AMPA, the mortality rates remained higher in SHRs (between 20% and 25%) compared with WKYS (no mortality observed) at all the ages analyzed. In these studies, MAP was significantly higher in 14- and 25-week-old SHRs compared with age-matched WKYS, but not in 7-week-old SHRs compared with age-matched WKYS (Figure 3A and 3B). The striatal damage induced by AMPA (2.5 nmol) in R-WKYS was not significantly different from normotensive WKYS (5±4 mm³ and 4±3 mm³, n=7 and 8, respectively; Figure 3C), despite a significant maintained increase in MAP in R-WKYS (Figure 3C). The intrastriatal administrations of AMPA (2.5 and 5 nmol) failed to modify the physiological parameters analyzed (data not shown).

Expression of AMPA Receptors Subunits

Measurements of mRNA by real-time polymerase chain reaction in the striata of adult WKYS (n=3) and SHRs (n=3) indicated that the levels of GluR1 and GluR2 are similar in both strains (Figure 4A and 4B). Protein expression analysis confirmed the lack of GluR1 and GluR2 subunit variations in both SHRs (n=3) and WKYS (n=3; Figure 4C and 4D).
Moreover, we have demonstrated by Western blotting, through the use of a selective antibody against the phosphorylated form of Ser831, that the level of pGluR1 is significantly higher in SHRs (n=3) when compared with WKYs (n=3; P<0.05; Figure 5A).

**Expression and Inhibition of CaMKII**

We observed that SHRs (n=3) express a higher level of CaMKIIα protein in the striatum when compared with WKYs (n=3; P<0.002; Figure 5B). Similarly, real-time polymerase chain reaction analyses showed that CaMKIIα mRNA level is higher in SHRs versus WKYs (data not shown). To further examine if the overexpression of CaMKIIα is involved in the exacerbation of brain damage in SHRs, 14-week-old rats were subjected to an injection of KN-93, an inhibitor of CaMKII, known to reduce the phosphorylation of GluR1.18,19 The intrastriatal administration of KN-93 resulted in 44% (P<0.01 vs vehicle; n=5) decrease in AMPA-induced brain damage in SHRs (n=8) but had no effect on the lesion in WKYs (n=7; Figure 5C).

**Discussion**

The present studies provide new evidence that SHRs display a specific sensitivity to AMPA, but not to NMDA, receptors activation. This sensitivity is related to a concomitant increase in the amount phosphorylated form of GluR1 at Ser831 and the CaMKIIα. The inhibition of this kinase reduced significantly the deleterious actions of AMPA receptors overactivation in SHRs, which suggest that the alteration of AMPA receptors phosphorylation may explain, at least in part, the vulnerability of this strain of rats to stroke, a condition in which excessive stimulation of glutamate receptors occurs.

SHRs are commonly used in preclinical investigations of stroke to integrate the major risk factor for the occurrence and the severity of stroke in man. Indeed, it has been shown that these rats, at adult age, display an exacerbated ischemic lesion in comparison to normotensive rats.2,20 This has been ascribed to the vascular alterations induced by the arterial hypertension that worsen blood perfusion after an occlusion.2 However, some reports suggest that these hypertensive rats display vulnerability of the cerebral parenchyma independently from the elevation of arterial pressure. Our results, showing that 7-week-old SHRs also exhibit an exacerbated ischemic damage when compared with age-matched WKYs, support this idea and concord with those reported in SHRs21 and in the other strain of SHRs that are stroke-prone.22 Moreover, through the use of the laser Doppler technique, we were able to verify that both normotensive and hypertensive rats displayed the same degree of severity of ischemia after MCAO. Nonetheless, it is worth mentioning that this technique allows measurements of relative perfusion only in a limited volume of the cortex. Accordingly, the CBF data merit completion with quantitative autoradiographic measures in the whole brain (eg, iodo-antipyrine).23,24 All the data discussed tend to suggest that, apart from collateral supply impairments described in hypertensive rats,21 other mechanisms, independent from this vascular component, will contribute to increase brain damage resulting from cerebral ischemia.
In an attempt to further analyze if there is an intraparenchymal vulnerability of SHRs to brain lesion, we focused on the effects of the overactivation of glutamatergic receptors in SHRs and WKYs through the use of a model of cerebral lesion induced by local administration of excitotoxins into the striatum, a brain structure known to be one of the most vulnerable to ischemic stress induced by MCAO. We show that the stimulation of NMDA receptors did not result in greater excitotoxic damage in hypertensive rats (SHRs and R-WKYs) compared with normotensive WKY rats. These data are particularly pertinent in the context of the pathogenesis of cerebral ischemia because, paradoxically, NMDA receptors are usually stated as having a predominant role in the neuronal death after stroke. In contrast, the stimulation of AMPA receptors induced a larger lesion and higher mortality rate in SHRs compared with WKYs. This exaggerated necrosis was independent from the age and the severity of hypertension, because this effect was found in 7-, 14-, and 25-week-old SHRs. To test whether these differences in AMPA vulnerability are strictly caused by inherent genetic differences between SHRs and WKYs or related to the level of MAP, we used the model of nongenetic hypertension, induced by renal artery stenosis in rats. This model provides further evidence that the level of MAP per se is not at the origin of the aggravation of the lesion induced by intracerebral AMPA administration. This specific vulnerability to AMPA but not to NMDA in SHRs is also consistent with previous neuroprotective studies in SHRs in which several AMPA receptors antagonists were efficient in terms of a significant decrease in ischemic brain damage, whereas NMDA receptor antagonists failed to protect the brain against ischemic injury in SHRs compared with normotensive rats.

Our hypothesis to explain the aggravation of AMPA-induced lesion in SHRs is a modulation of AMPA receptor functions in SHRs. This hypothesis is supported by the literature available for glutamatergic receptors expression in SHRs. Several studies demonstrate increases of the expression of AMPA receptors subunits (GluR1 and GluR3) in the nucleus of the solitary tract in SHRs and also an enhanced glutamate activation in the prefrontal cortex of SHRs compared with WKYs through a mechanism that involves AMPA, but not NMDA, receptors. AMPA receptor properties depend on the combination of their 4 subunits, GluR1 to GluR4. AMPA receptors that possess the GluR2 subunit exhibit low calcium permeability.
ity, whereas the other subunits are thought responsible for high calcium permeability.31 After stroke, excessive release of glutamate leads to a deleterious influx of calcium in neurons, especially via AMPA receptors. Here, we show, by both quantitative measurements of mRNA and protein expression, that there is no difference in GluR1 and GluR2 subunits in the striata of SHRs and WKYs. The Ca\(^{2+}\) permeability associated with AMPA receptors is linked not only to subunits proportions but also to modifications in the subunits properties. One of the main mechanisms implicated in the modulation of AMPA activity is the phosphorylation of GluR1, which leads to

![Western blot](image.png)

**Figure 5.** Expressions of pGluR1 and CaMKII\(\alpha\) proteins in the striata of 14-week-old WKYs and SHRs and inhibition of CaMKII\(\alpha\) by KN-93. Western blotting analyses show that pGluR1 at Ser831 (A) and CaMKII\(\alpha\) (B) are significantly increased in SHRs compared with WKYs. Analyses were performed on total protein samples (20 \(\mu\)g) from 3 animals of both strains (numbered 1 to 3). The pGluR1 and CaMKII\(\alpha\) protein expressions are normalized to total GluR1 and GAPDH expressions, respectively. Data are presented as mean±SD. C, KN-93 significantly reduced the volume of AMPA-induced lesion in SHRs. KN-93 (0.4 mmol/L) or vehicle (saline, \(n=5\)) was administered into the striatum 30 minutes before the injection of AMPA. Results are expressed as mean±SD. **\(p<0.002\) compared with saline-treated SHR, ††\(p<0.01\) compared with saline-treated SHRs (Student \(t\) test).
increase up to 50% the conductance of AMPA phosphorylated receptors. In our studies, we demonstrate that SHR s display significantly higher levels of the phosphorylated form of GluR1 in the striatum compared with WKYs. Overall, our data suggest that the susceptibility of SHRs to AMPA is not associated with the genetic alterations of the expression of AMPA receptors subunits GluR1 and GluR2 but rather to posttranslational modifications leading to increased forms of phosphorylated GluR1. We propose that this exacerbation of AMPA receptors permeability in SHRs provokes a dramatic increase in cellular death in pathological condition.

Several modulators of GluR1 activity have been identified and of particular interest is the CaMKIIα, which is a ubiquitous signaling molecule, found in high concentrations in neurons. The privileged substrate of CaMKII is a ubiquitous signaling molecule, found in high concentrations in neurons. Here, we show for the first time that CaMKIIα is overexpressed in the striata of SHRs compared with WKYs, which might explain the exacerbation of the brain lesion in those hypertensive animals. This idea is supported by the observed reduction in AMPA-induced excitotoxic brain damage in SHRs after the administration of KN-93, an inhibitor of CaMKIIα. Further indication of the role of the CaMKIIα could be provided by the study of the effect of KN-93 in cerebral ischemia; such an investigation has not been performed yet, but some data suggest a potential beneficial effect.

An appealing perspective arising from our findings of the CaMKIIα overexpression in SHRs is to determine its potential significance in the hypertensive pathology. To further investigate this point, more studies still need to be undertaken to examine the AMPA receptors modulation in both animals with long-term hypertension and in other strains of hypertensive models. Such studies would allow us to determine how this specific phenotype could be related to hypertension and stroke sensitivity.

In conclusion, our present study shows that the exacerbation of damage after stroke in SHRs is not solely dependent on the level of arterial hypertension. SHRs also display a hyperpolarizability to AMPA-induced brain damage linked to an increased phosphorylation of GluR1 in the striatum. These findings need to be taken into account in future studies of the pathophysiology and the treatment of focal stroke in which SHR is used. This strain of rats is commonly used in preclinical investigations of stroke to integrate the major risk factor for the occurrence and the severity of stroke in humans.

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Disclosures
None.

References


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