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Reduction of cerebral injury in stroke-prone spontaneously hypertensive rats by amlodipine

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Abstract

Dihydropyridine Ca^{2+} channel antagonists, initiated together with high salt intake, prevent the development of hypertension and subsequent cerebral damage in stroke-prone spontaneously hypertensive rats (SHRSP). We hypothesized that the dihydropyridine Ca^{2+} channel antagonist amlodipine (approximately 15 mg/kg/day) could also reverse established hypertension and cerebral damage. SHRSP drank 1% NaCl from 8 weeks of age. Cerebral damage (cerebral edema and blood–brain barrier integrity) was investigated with magnetic resonance imaging twice a week. Systolic blood pressure was measured weekly. All rats developed severe hypertension and subsequent cerebral damage (defined as day 0). Untreated controls ($n=7$) died at day 12 (range: 7–28). Oral treatment with amlodipine ($n=7$), initiated at day 0, reduced systolic blood pressure, reversed cerebral edema and restored blood–brain barrier integrity. Systolic blood pressure remained low and eventually rats died after 450 days (range: 350–580) showing nephrosis but no recurrence of cerebral damage. In conclusion, established hypertension and cerebral damage are reversed by amlodipine in SHRSP.

Keywords: Cerebral edema; Dihydropyridine Ca^{2+} channel antagonist; Magnetic resonance imaging; Hypertension; Hypertensive encephalopathy; (Rat)

1. Introduction

Stroke-prone spontaneously hypertensive rats (SHRSP), subjected to high NaCl intakes, show severe hypertension, encephalopathy, glomerulopathy, and early death (Nagaoka et al., 1976). Previously, we found that angiotensin-converting enzyme inhibition and angiotensin AT_1 receptor blockade could reverse cerebral injury in salt-loaded SHRSP when treatment was started after manifestation of cerebral edema (Blezer et al., 1998, 2001). However, within 1 year cerebral injury recurred, despite continued treatment, in most of the rats. Reversal of injury by angiotensin-converting enzyme inhibition and angiotensin AT_1 receptor blockade occurred without a persistent decrease in blood pressure. It is conceivable that unabated severe hypertension leads to the recurrence of cerebral injury. Preventive treatment with dihydropyridine Ca^{2+} channel antagonist in SHRSP started simultaneously with high NaCl intake reduces blood pressure and organ damage, and prolongs survival (Shinyama et al., 1995; Kyselovic et al., 1998). Generally, dihydropyridines are more effective than angiotensin-converting enzyme inhibitors in lowering blood pressure in hypertensive blacks (Cappuccio et al., 1993; Radevski et al., 1999), especially when salt-intake is high (Weir et al., 1998). In black patients with hypertensive crisis, the slow-acting dihydropyridine (nifedipine-retard) causes a more effective blood pressure fall than an angiotensin-converting enzyme inhibitor (captopril), without the rapid fall seen after regular dihydropyridines (nifedipine) (Damasceno et al., 1997). Shortly before the occurrence of hemorrhagic stroke, SHRSP lose their ability to autoregulate cerebral blood flow (Smeda et al., 1999). It is conceivable that slow-acting dihydropyridines act favorably on hypertensive encephalopathy, because of the combined effect of gradual, but marked, blood pressure reduction and at least partial maintenance or recovery of cerebral autoregulation. Thus, we hypothesized that the dihydropyridine

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Ca^{2+} channel antagonist amlodipine would reverse the cerebral edema occurring in salt-loaded SHRSP, and that this would probably be accompanied by a marked reduction in blood pressure.

Hence, we tested whether in salt-loaded SHRSP with established cerebral injury, oral treatment with amlodipine could reverse such hypertensive encephalopathy, in conjunction with a marked reduction in blood pressure. The extent of hypertensive encephalopathy was assessed non-invasively using T_2-weighted Magnetic Resonance Imaging (T_2WMRI) to quantify cerebral edema and Gadopentetate dimeglumine enhanced T_1WMRI to quantify protein leakage through the blood–brain barrier (Naruse et al., 1982).

2. Materials and methods

2.1. Animals

Male SHRSP ($n = 14$), aged 6 weeks, were obtained from IFFA Credo, France. They were housed in constant environmental conditions (12-h light/dark; humidity 55%; temperature 22 °C), were given free access to a standard rat chow (RMH-TM rat chow; protein 22.2%; fat 4.8%; K^+ 0.85%; Na^+ 0.40%; Hope-Farms, Woerden, The Netherlands) and allowed water ad libitum. The Utrecht University Committee for study in experimental animals approved the protocol.

2.2. Protocol

Baseline measurements were done in all rats at 7 weeks of age. Subsequently, at the age of 8 weeks, all rats were switched to drinking water with a high NaCl content (1% NaCl weight/weight), i.e. 170 mmol/l. This accelerates the appearance of cerebral edema (Nagaoka et al., 1976). The rats were observed daily for overt neurological symptoms and underwent weekly blood pressure measurements and 24-h urine collection.

Rats were subjected to the high salt intake continuously, and subjected to MRI every 3–4 days after proteinuria exceeded 40 mg/day, until the detection of the first cerebral abnormalities. At this point, they alternatively entered one of two groups. Group 1 served as a control ($n = 7$). In group 2 ($n = 7$), amlodipine (50 mg/l) was added to the drinking water after the first observation of cerebral edema with T_2WMRI. This resulted in an intake of amlodipine of 7–20 mg/kg/day during the first 392 days of treatment (range: 252–462 days). Thereafter, intake increased due to polydipsia because of renal failure. Rats from group 1 were subjected to MRI every 3–4 days until the experiment was stopped when an animal was very debilitated (hemiparalysis, severe myoclonia, or ataxis and cachexia), or died spontaneously. The rats from group 2 were subjected to MRI at days 3, 7, 10, 14, 21 and 252 after the start of treatment. Finally, T_2WMRI of formalin-fixed brains was performed after spontaneous death.

2.3. Blood pressure and proteinuria

Systolic blood pressure was measured with tail-cuff plethysmography (IITC, San Diego, CA, USA) weekly in the conscious rats after prewarming. Urinary protein was determined with Coomassie Blue.

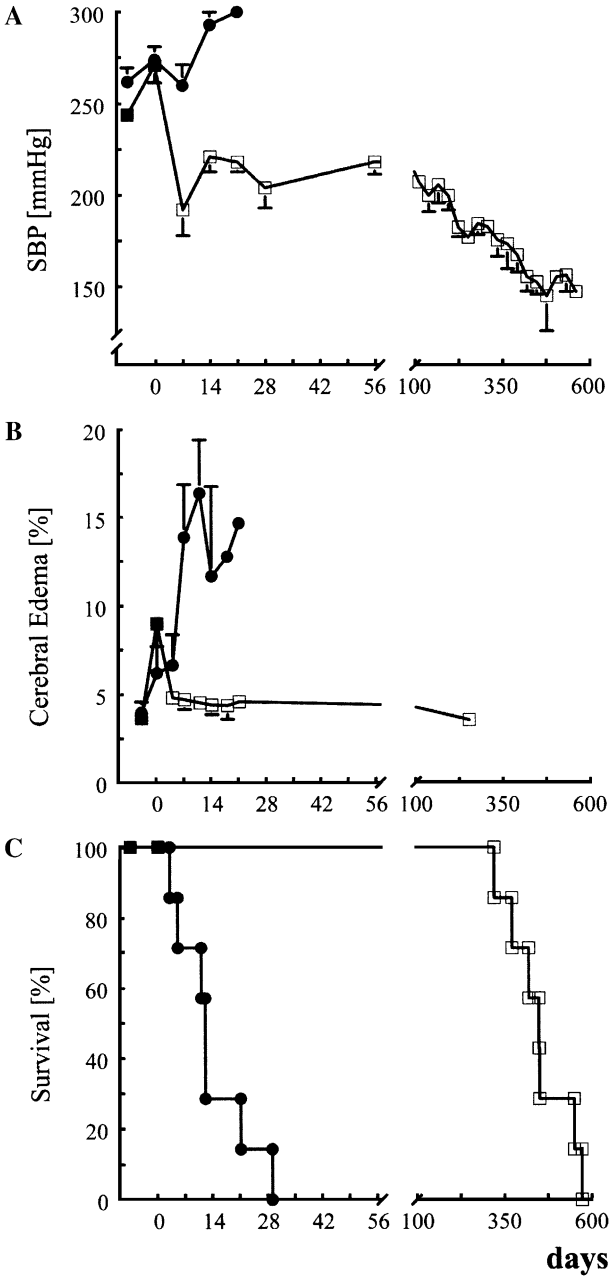

Fig. 1. (A) Systolic blood pressure (systolic blood pressure, mm Hg) in control SHRSP (●) and in SHRSP where long-term treatment with amlodipine was initiated after the first detection of cerebral edema (before treatment: ■; after treatment: □). (B) Cerebral edema (% pixels). (C) Survival (%).

2.4. MRI

Anesthesia was induced with a mixture of fentanyl citrate (0.315 mg/kg), fluanisone (10 mg/kg) and midazolam (5 mg/kg). Rats were intubated and mechanically ventilated with 1% halothane in N_2O/O_2 (70%/30%). A tail vein was cannulated for applying a bolus of 1-deoxy-1-(methylamino)-D-glucitol dihydrogen [*N*,*N*-bis[2-[bis(carboxymethyl)amino]ethyl]-glycinato-(5-)]gadolinate (2-) (2:1) (Gadopentetate dimeglumine, Magnevist®, Schering, Berlin, Germany). During the MRI-session, mechanical ventilation continued and expiratory CO_2 was continuously monitored. Body temperature was maintained at 37 °C with a heated water pad. The animals were fixed in a stereotaxic holder to prevent movement and positioned in a 4.7-T, 200 MHz Nuclear Magnetic Resonance-spectrometer (Varian, Palo Alto, CA, USA) with a high-performance gradient insert (maximum gradient strength 220 mT/m). A 120-mm Helmholtz coil was used for both transmission and signal reception. After the sagittal scout image, the following coronal MRI images were collected (for all: matrix 128 × 128; zero filled to 256 × 256; FOV 40 × 40 mm): T_2 W images (25 slices × 1 mm; repetition time = 3000 ms; echo time = 30 ms; nt = 2) and pre and post (bolus of 0.5 mmol Gadopentetate dimeglumine/kg, 20 min in circulation) contrast enhanced T_1W images (13 slices × 1.5 mm; repetition time = 375 ms; echo time = 28 ms; nt = 4).

2.5. MRI data evaluation

2.5.1. T_1-weighted images

A gadolinium-enhancement ratio (GER) image was calculated from the pre and post contrast Gadopentetate dimeglumine enhanced images according to

$$\mathrm{GER} = [(T_1\mathrm{WMRI}_{\text{post contrast}} - T_1\mathrm{WMRI}_{\text{pre contrast}}) / T_1\mathrm{WMRI}_{\text{pre contrast}}] \times 100 \quad (1)$$

Pixel-intensities thus display the % signal increase due to Gadopentetate dimeglumine leakage. At day 0, the area that showed the primary leakage of Gadopentetate dimeglumine was determined. In this area, and in the same area of the two adjacent slices, a square region of interest (0.35 mm^2) was placed in which the mean intensity was calculated. The

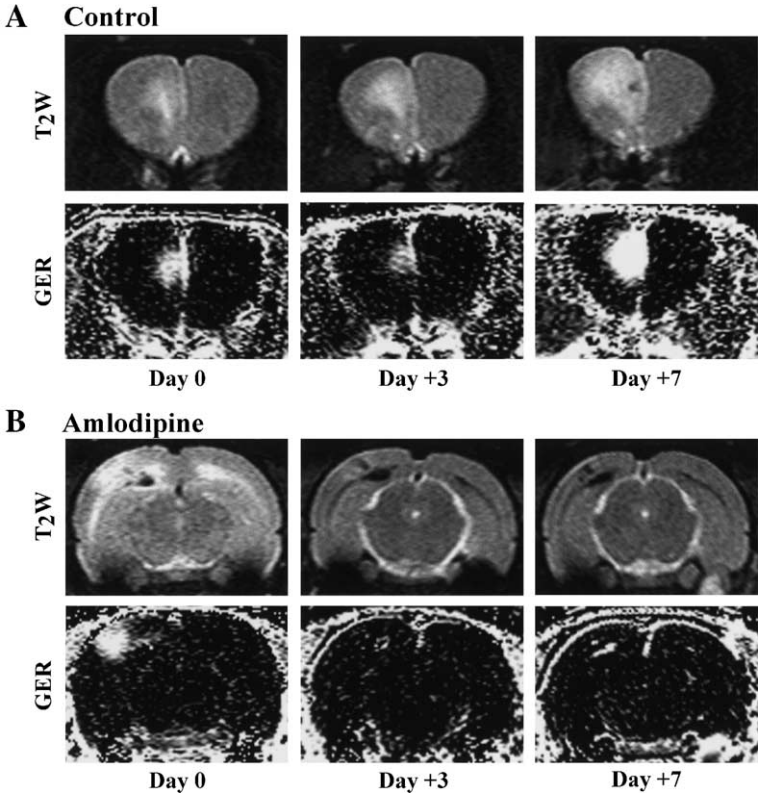

Fig. 2. (A) Upper row: typical MR images of progression of cerebral injury in control SHRSP in the first week after detection of cerebral edema (day 0). T_2WMRI shows an expanding area affected with cerebral edema with a hemorrhagic focus (i.e. hypointensity) appearing on day 7. Lower row: typical GER images (calculated from pre and post contrast Gadopentetate dimeglumine enhanced images) of progression of leakage through the blood–brain barrier in control SHRSP in the first week after detection of leakage (day 0). (B) Upper row: typical MR images of reduction of cerebral injury in amlodipine-treated SHRSP in the first week after detection of cerebral edema (day 0). T_2WMRI shows that cerebral edema had disappeared within 3 days. An area affected by hemorrhage on day 0 remains visible. Lower row: typical GER images of regression of leakage through the blood–brain barrier in amlodipine-treated SHRSP within 3 days after detection of leakage (day 0).

same calculations were done in the corresponding region of interest at day −3 and day +3.

2.5.2. T_2-weighted images

The amount of cerebral edema in the T_2W images was determined according to methods described previously (Blezer et al., 1998) in which pixels above a defined threshold, i.e., mean + 2 × standard deviation of the pixel intensity in the unaffected situation, were defined as edematous.

2.6. Pathology

Brains were collected in buffered formaldehyde for histology. After paraffin embedding, 10 μm serial sections (10 sections at 500 μm intervals) were cut. Staining was done with hematoxylin–eosin (HE).

2.7. Statistics

Data were evaluated by two-way ANOVA for repeated measurements, followed by a multiple comparison procedure (Student–Newman–Keuls Method). Data are presented as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

3. Results

All rats developed severe hypertension (Fig. 1A) and proteinuria before the occurrence of cerebral edema (Fig. 1B). This was accompanied by unifocal leakage of Gadopentetate dimeglumine through the blood–brain barrier (Fig. 2A). Controls died 12 days (range: 7–28) after appearance of cerebral edema (Fig. 1C) and showed gross cerebral lesions (Fig. 3A and 3C). On day 0 in the controls systolic blood pressure was 274 ± 7 mm Hg and proteinuria was 298 ± 46 mg/day, values very close to those observed before the start of amlodipine treatment in group 2 (see below). Thus, there was no bias in the selection of rats for one of the two groups.

Amlodipine caused a dramatic increase in survival, all rats surviving for nearly 1 year after the appearance of cerebral edema (Fig. 1C). Median survival after the appearance of cerebral edema increased to 450 days (range: 350–580). Body weight also increased steadily, after an initial decrease directly before the initiation of treatment. Amlodipine reduced systolic blood pressure from 273 ± 9 mm Hg on day 0 to 187 ± 11 mm Hg on day 7 ($P < 0.05$). Although systolic blood pressure increased to about 210 mm Hg from day 14 to day 56 it subsequently fell to about 180 mm Hg by day 224 and continued to fall in the surviving rats (Fig. 1A). Amlodipine reduced proteinuria from 298 ± 60 mg/day on day 0 to 79 ± 16 mg/day on day 7 ($P < 0.05$). Amlodipine-treated rats died showing severe nephrosis, with visceral edema and massive proteinuria (1086 ± 142 mg/24 h).

Amlodipine caused a direct reduction in cerebral edema and blood–brain barrier leakage (Fig. 2B). Edema decreased from 9.0 ± 2.7% pixels on day 0 to 4.8 ± 0.5% pixels on day 7 ($P < 0.05$). Cerebral edema was still at this low level at day 252 (Fig. 1B). Three days after initiation of amlodipine treatment, Gadopentetate dimeglumine leakage, and thus GER was reduced from 25.5 ± 3.4% to 20.0 ± 0.3% ($P < 0.05$), a level similar to that observed 3 days before the occurrence of Gadopentetate dimeglumine leakage (19.6 ± 0.7%). Therefore blood–brain barrier integrity,

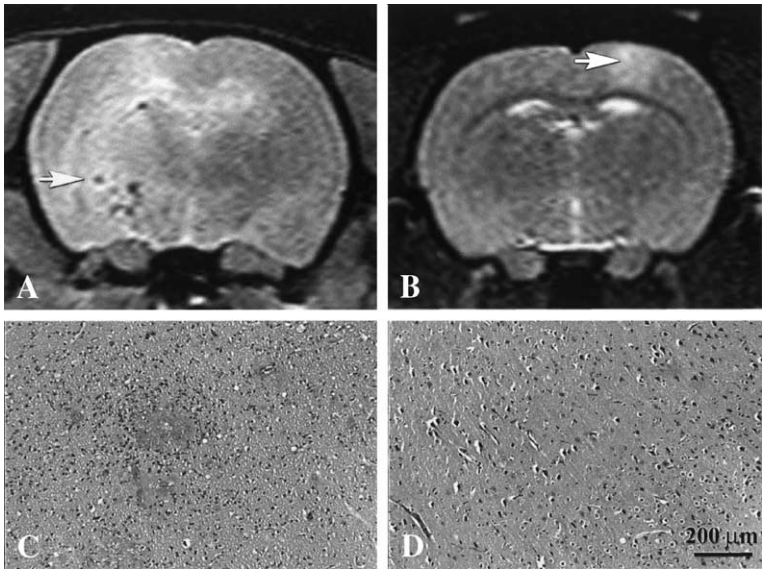

Fig. 3. (C) Histology section of the hyperintense area of the control SHRSP (arrow in T_2W image in panel A; control SHRSP with terminal cerebral injury on day 3), extracellular vacuoles and focal hemorrhage can be observed. (D) Absence of gross injury in the area previously affected by cerebral edema (arrow in T_2W image in panel B; amlodipine-treated SHRSP showing initial injury on day 0) in the amlodipine-treated SHRSP on day 433.

as evaluated by MRI techniques, was restored. No signs of cerebral injury were observed during the treatment period, and no focus of cerebral edema was observed postmortem by T_2WMRI and histology in any of the amlodipine-treated rats (Fig. 3B and 3D).

4. Discussion

The main and novel finding of this study was that in a rodent model of severe hypertension accompanied by cerebral damage, gross protein leakage through the blood–brain barrier can be halted by the dihydropyridine L-type Ca^{2+} channel antagonist amlodipine (7–20 mg/kg/day). Reversal of this injury by amlodipine was accompanied by a reduction in blood pressure.

A high salt intake suppresses blood pressure dependency on the renin–angiotensin system. Indeed, in this model angiotensin-converting enzyme inhibition or angiotensin AT_1 receptor blockade did not have a sustained antihypertensive effect (Blezer et al., 1998, 2001). In contrast, Ca^{2+} channel antagonists decreased blood pressure. Under conditions of NaCl loading such an antihypertensive effect is to be expected. In the salt-loaded SHRSP, Ca^{2+} channel antagonism probably facilitates Na^+ excretion by the kidney so that a lower blood pressure can be maintained (Guyton et al., 1980). This has indeed been shown in this model in which dihydropyridines increased urine volume and urinary excretion of Na^+ (Ichihara et al., 1998). A similar antihypertensive effect has been observed in salt-loaded SHRSP with the long-acting Ca^{2+} channel antagonist, lacidipine (Kyselovic et al., 1998).

It is known that SHRSP have serious cerebral microcirculatory disturbance before or at acute stroke (Zhai and Duan, 1993). SHRSP lose their ability to autoregulate cerebral blood flow prior to stroke and cerebral blood flow increases with increasing blood pressure. This enhanced cerebral perfusion (Smeda et al., 1999) could eventually lead to the appearance of hypertensive encephalopathy induced vasogenic edema. It is suggested that this edema could, if untreated, increase intracranial pressure, resulting in a reduction of the cerebral blood flow (Funato et al., 1999), and the formation of cerebral hemorrhage (Smeda et al., 1999).

Cerebral edema, and therefore presumably blood–brain barrier protein leakage, was reversed by amlodipine. This occurred within 4 days, at first glance suggesting direct beneficial effects of blood pressure lowering. However, equally rapid reduction of cerebral edema was also observed after starting angiotensin-converting enzyme inhibition or angiotensin AT_1 receptor blockade without much change in blood pressure (Blezer et al., 1998, 2001). Several studies in salt-loaded SHRSP show that prevention (Shinyama et al., 1995; Ueda et al., 1993) or reduction (Funato et al., 1999; Ueda et al., 1993; Shinyama et al., 1998; Ueno et al., 2000) of stroke signs by initiation of dihydropyridines is accompanied by a reduction in blood pressure. However, other studies in this model show ameliorating effects of Ca channel blockers without a profound reduction in systolic blood pressure (Kyselovic et al., 1998; Feron et al., 1995). It has been suggested that Ca channel blockers directly improve the structural integrity of the blood–brain barrier by reducing the endothelial permeability (Nag, 1991). Other non-hemodynamic beneficial effects of Ca channel blockers may be due to direct protective effects on central neuronal cells (Gemba et al., 1993), improvement of cerebral circulation by dilatation of microvessels (Zhai and Duan, 1993), blockade of the effects of growth-factors (like angiotensin) on vascular endothelial cells (Sachinidis et al., 1992), correction of abnormal sensitivity of arterial smooth muscle cells to Ca^{2+} (Jiang et al., 1995), and directly inhibiting the detrimental effects of oxidative processes induced by oxygen radicals (Napoli et al., 1999). It is conceivable that both hemodynamic and non-hemodynamic actions contributed the marked and long-lasting beneficial effects of amlodipine in our model.

In patients, a too rapid reduction in blood pressure in the presence of cerebral injury should be avoided because of the risk of accelerating impairment of cerebral circulation by the steal phenomenon (Strandgaard and Paulson, 1990) or by inducing autoregulatory dysfunction (Gaab et al., 1990). It is reasonable to assume that, autoregulation of cerebral blood flow is maintained during chronic dihydropyridine (amlodipine)-treatment in the SHRSP. It has been shown in SHR that a reduction of the mean arterial pressure was accompanied by a shift of the lower limit of the autoregulation curve to the left (Cai et al., 1996. Moreover, dihydropyridine treatment in patients with severe hypertension gives a smooth blood pressure reduction without general or focal cerebral hyperemia (felodipine: Thulin et al., 1993; amlodipine: Pandita-Gunawardena and Clarke, 1999). Interestingly, amlodipine had a hypotensive effect and caused remission of neural symptoms in severely hypertensive cats with encephalopathy accompanying spontaneous chronic renal disease (Henik et al., 1997), implying that amlodipine may also have beneficial actions in non-rodent models of hypertensive cerebral damage.

A novel aspect in our study was the application of quantitative analysis of cerebral edema (T_2WMRI) plus blood–brain barrier leakage (GER). This approach revealed a linear correlation ($r = 0.74$; $P < 0.01$) between these two variables suggesting that increased vascular leakage rather than a reduced clearance of extravascular fluid was the cause of cerebral edema, and that amlodipine was improving vascular leakage rather than accelerating the clearance of leaked fluid. Of course, the resolution of MRI has limitations and microscopic multifocal blood–brain barrier leakage (Fredriksson et al., 1988) may already have occurred before we observed the macroscopic unifocal lesion with MRI. Cerebral injury did not recur in the amlodipine-treated rats, at least not to the extent that we could detect it. This is in contrast to what we observed after an angiotensin-converting enzyme inhibition or angiotensin AT_1 receptor blockade where extensive cerebral injury recurred shortly

before death in 75% of the rats that had initially been affected (Blezer et al., 1998, 2001). Possibly, the approximately 100-mm Hg difference in systolic blood pressure between the rats in the present study and the rats in those studies underlies this difference.

In conclusion, this study shows that amlodipine can cause reduction of cerebral damage and considerably prolong survival, in a rodent model of genetic predisposition to hypertensive encephalopathy accelerated by high salt intake. These findings may be relevant for hypertensive patients with an analogous predisposition and exposure to high levels of dietary Na^+.

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