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Calcium antagonists decrease capillary wall damage in aging hypertensive rat brain

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Abstract

Chronic hypertension during aging is a serious threat to the cerebral vasculature. The larger brain arteries can react to hypertension with an abnormal wall thickening, a loss of elasticity and a narrowed lumen. However, little is known about the hypertension-induced alterations of cerebral capillaries. The present study describes ultrastructural alterations of the cerebrocortical capillary wall, such as thickening and collagen accumulation in the basement membrane of aging spontaneously hypertensive stroke-prone rats. The ratio of cortical capillaries with such vascular pathology occurred significantly more frequently in hypertensive animals.

Nimodipine and nifedipine are potential drugs to decrease blood pressure in hypertension but their beneficial effects in experimental studies reach beyond the control of blood pressure. Nimodipine and nifedipine can alleviate ischemia-related symptoms and improve cognition. These drugs differ in that nifedipine, but not nimodipine reduces blood pressure at the here-used concentration while both drugs can penetrate the blood-brain barrier. Here we show that chronic treatment of aging hypertensive stroke-prone rats with nimodipine or nifedipine could preserve microvascular integrity in the cerebral cortex. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Hypertension; Cerebral capillary; Ultrastructure; Calcium channel antagonist; Cerebrovascular protection

1. Introduction

Chronic hypertension has a profoundly detrimental effect on cerebral circulation and brain function. For experimental approaches to study hypertension, the spontaneously hypertensive stroke prone rat strain (SHR-SP) proved to be an apt animal model to investigate the consequences of prevailing hypertension on behavior, neuronal integrity and cerebrovascular parameters. Preliminary studies demonstrated that chronic hypertension in 60 weeks old SHR-SP evoked microvascular pathology such as capillary basement membrane thickening and collagen deposits in the basement membrane [14,15]. Interestingly, similar cerebrovascular malformations and related behavioral impairment were reported in normotensive, senescent rats [6–9,35], which support the theory that chronic hypertension accelerates the progressive aging-related deterioration of the central nervous system. In addition, both in hypertensive and in aged

animals it was observed that cognitive decline coincided with changes in neuronal calcium homeostasis. Studies employing the L-type calcium channel antagonist nimodipine demonstrated that nimodipine could remarkably moderate not only signs of neurodegeneration as a consequence of hypertension or aging, but also the aging-related microvascular abnormalities [6–9,35,36].

Preliminary findings on morphological microvascular changes in SHR-SP [14,15] raised further questions. First, is the severity of the pathological changes in capillaries related to the age of the experimental animals? Second, does cerebral microvascular breakdown directly stem from chronic hypertension or is it caused by a neurogenic derangement of calcium homeostasis secondary to hypertension? To resolve these questions, we analyzed the cerebral capillary ultrastructure of two age groups (40 weeks old and 60 weeks old) of hypertensive and normotensive rats. Furthermore, we applied a long-term treatment of two dihydropyridine type calcium channel blockers nimodipine and nifedipine on additional experimental groups.

The use of dihydropyridine type calcium channel blockers as anti-aging treatment was based on the finding that

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calcium influx through voltage sensitive calcium channels appeared to be a major contributor to the dysregulation of neuronal calcium concentration during aging [11,36,44]. The typical, aging-related decline of neural functions (memory capacity, sensorimotor functions) can stem from the imbalance of the intracellular calcium homeostasis which became a target of pharmacological treatment [44]. Nimodipine and nifedipine can protect cellular integrity by binding to the L-type calcium channels thus blocking the potentially harmful calcium influx to cells. We aimed at making use of the unique properties of the two drugs that they penetrate the blood-brain barrier (BBB) [25,32,50] and can act on neural elements [46]. Nimodipine does not affect blood pressure, while nifedipine lowers blood pressure in the here used concentration. Based on the treatments, we evaluated the ultrastructural lesions of the cerebral capillary basement membrane in the frontal cortex of 60 weeks old SHR-SP rats and examined the potentially protective effects of the drugs on the cerebral microvasculature.

2. Materials and methods

The study was designed to test the effect of aging and the potentially beneficial effects of two L-type calcium channel antagonists, nifedipine and nimodipine on the ultrastructure of cerebral capillaries in male hypertensive rats (SHR-SP, breeder: Møllegaard, Skovøvnd, Denmark). In order to assess age-related vascular malformations, four groups of rats were used. These groups were the following: 40-week old SHR-SP rats (SP-40; $n = 6$), 40-week old Wistar Kyoto rats (WKY-40; $n = 6$), 60-week-old WKY rats (WKY-60-plac; $n = 6$) and 60-week-old SHR-SP rats (SP-60-plac; $n = 8$). The WKY animals served as normotensive controls. The animals were group-housed, lived under a standard 12 h light-dark cycle and had access to water and food *ad libitum*.

The behavioral profile of the animals was characterized by a small open field test, a novelty-induced behavioral arousal paradigm. The rats were individually placed into a new cage ($24.5 \times 24.5 \times 30$ cm) and were observed for 60 min on week 40 (WKY-40, SP-40) or week 60 (WKY-60, SP-60). The actual behavioral activity pattern they were displaying (rearing, grooming, sniffing, walking, immobility) was registered every 10 s. A behavioral score was calculated for each kind of explorative activity which reflected how often the animal showed that particular behavior in the frame of the 60 min observation time.

The systolic blood pressure values (SBP) of groups WKY-40 and SP-40 were taken on week 40 before sacrificing the animals. The SBP of groups WKY-60-plac and SP-60-plac were repeatedly measured on every fourth week between the age of 40 and 60 weeks. The SBP values were assessed with the tail-cuff method on animals slightly anesthetized by ether. Then the rats were anesthetized by an i.p. overdose of sodium pentobarbital and sacrificed by a trans-

cardiac perfusion of physiological saline containing 1% heparin followed by a solution of 1% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were stored in 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB at 4°C until further processing.

Other groups of WKY and SP animals running parallel with the above WKY-60-plac and SP-60-plac groups received either a nifedipine or a nimodipine treatment for 20 weeks between the age of 40–60 weeks. The compounds were mixed in the regular rat chow. The daily drug-intake of the animals was defined as 20–25 mg. The small open field test of novelty induced exploration was performed together with the above mentioned four groups. SBP was also measured on every fourth week and the last value was used for later correlation analysis. Three groups of SHR-SP rats were used: 6 animals were fed with nifedipine-containing chow (SP-60-nife) and 6 animals with nimodipine-containing food (SP-60-nimo). Each of these groups had their normotensive WKY controls, (WKY-60-nife, $n = 6$; WKY-60-nimo, $n = 6$). The food and water intake of the animals, as well as their body weight was regularly monitored to verify comparable drug intake of the separate groups. Because of group housing, the food and water intake of individual animals was calculated by dividing the absolute amount of food and water consumed in a cage by the number of animals in the cage. At the age of 60 weeks, the animals were anesthetized, perfused, and their brains post-fixed as described above.

Brain slices were cut at 50 μ m with a vibratome, routinely dehydrated and embedded in glycid ether. Samples of the frontal cortex at 0.2 mm rostral to Bregma were then selected and mounted on glycid ether blocks. Non-serial, ultrathin sections were collected on 200 mesh copper grids and examined with a Philips 201 and a Philips CM 10 electron microscope.

One hundred capillaries per case were screened throughout the cortical layers focusing on the microvascular basement membrane (BM) and pericyte pathology as defined in detail previously [13]. Briefly, the following categories of capillary abnormalities were distinguished: local basement membrane thickening (BMT) if the luminal and abluminal outline of the BM were not running parallel at a given segment of the membrane, as shown in Fig. 1B. BMT was additionally characterized by measuring the thickness of the BM: the width of a healthy BM segment ranged between 50–100 nm while thickening typically varied between 150–550 nm. Fibrosis referred to excessive collagen type IV accumulation between two layers of the BM identified by its periodicity, or collagen invasion to the vascular pericytes from a split BM (Fig. 1C). When a capillary demonstrated both BMT and fibrosis, fibrosis overruled BMT and the capillary was counted only as one with fibrosis. Degenerative pericytes showed abnormal, membranous-appearing inclusion bodies or swelling (Fig. 1D). The region investigated did not show signs of cerebrovascular infarcts.

Fig. 1. Schematic drawings demonstrating the transversal profile of an intact cerebral capillary (A), local basement membrane thickening indicated by arrowheads (B), fibrosis pointed at by arrowheads (C) and pericytic degeneration (D). Abbreviations: e: endothelial cell, en: endothelial nucleus, l: capillary lumen, p: pericyte, pn: pericytic nucleus, *: basement membrane.

The density of capillaries was determined as the number of transversal capillary profiles counted on a standard surface area of 0.2 mm^2 spanning all cortical layers. The diameter of the capillary lumen of 15–24 microvessels of a standard surface area was also measured. The surface areas were standardized with the help of the grid mesh.

The data were statistically analyzed by the computer program SPSS. The quantitative results of capillary ultrastructure were calculated as percentages of the total number of vessels investigated, expressed as median values and evaluated using the Mann-Whitney-*U* test. SBP was analyzed by repeated measurement ANOVA, changes in capillary diameter and density by one-way-ANOVA while the correlation graphs were tested with the Pearson correlation test.

3. Results

The analysis of the novelty-induced exploration showed that two of the behavioral parameters, namely rearing and grooming, significantly differed between groups. Fig. 2A represents the frequency of rearing behavior. Age-related decrease of rearing could not be detected when comparing WKY-40 with WKY-60-plac or SP-40 with SP-60-plac but clear and significant reduction was seen in SP-40 and SP-60-plac when compared to their WKY controls. In addition,

nimodipine or nifedipine treatment could increase rearing behavior to normotensive levels. Grooming activity presented in Fig. 2B also appeared to be independent of age under normotensive conditions (WKY), but was considerably reduced in SP-60-plac compared to SP-40 and also to WKY-60-plac. Thus, the decrease in grooming activity in old SP could be associated with both age and hypertension. Nimodipine and nifedipine administration restored the grooming score to normotensive values in the SP-60 nimo and SP-60-nife groups.

The SBP values are presented in Fig. 3A & B. The SBP of the onset SP-40 group was about 60 mmHg higher than in WKY-40. During the experimental period (20 weeks) the SBP did not increase in any of the groups. Neither nimodipine, nor nifedipine administration altered the SBP of aging WKY animals while nifedipine—but not nimodipine—decreased the SBP in the aging SHR-SP animals already 1 month after the beginning of the treatment.

Fig. 4A & B is showing the food intake of the six 60 weeks old experimental groups while Fig. 4C & D is demonstrating the water intake throughout the calcium antagonist-treatment period. It is important to notice that comparing the WKY-60-nimo and WKY-60-nife groups, and the SP-60-nimo and SP-60-nife groups also with each other, the animals ate and drank the same amount indicating comparable drug intake. There was no difference in body weight among the WKY groups (Fig. 4E) but when comparing the

Fig. 2. Novelty-induced exploratory behavioral profile expressed as rearing frequency (A; *: $P < 0.05$, **: $P < 0.01$) and grooming frequency (B; *: $P < 0.05$) during 60 min observation.

SP groups, SP-60-nimo rats were significantly heavier than SP-60-plac animals as analyzed by repeated measurement ANOVA ($F = 7.156$, $*P < 0.02$). There was no difference between the SP-60 nimo and SP-60 nife groups (Fig. 4F).

Fig. 5 provides electron microscopic photomicrographs of microvascular degenerative features assessed in this study. The age-related changes in capillary integrity were assessed by comparing the 40 and 60 weeks old groups. Fig. 6 shows that the WKY-60-plac demonstrated an increased level of BMT, an unchanged degree of fibrosis, and a significantly higher incidence of pericytic degeneration when compared to WKY-40. When comparing SP-40 and SP-60-plac, the difference in capillary basement membrane pathology appeared to be more pronounced than that seen in the normotensive WKY rats. Capillaries with BMT occurred three times more frequently in the SP-60-plac than in the SP-40 and the ratio of fibrosis doubled. An increased percentage of microvessels with degenerating pericytes was also found, which was similar to the WKY groups.

Hypertension-related microvascular degeneration could

Fig. 3. Systolic blood pressure values measured in the onset groups (A; **: $P < 0.01$), and the dihydropyridine treated groups (B; *: $P < 0.05$ WKY-60-plac x SP-60-plac, \$: $P < 0.05$ SP-60-plac x SP-60-nimo, +: $P < 0.05$ SP-60-plac x SP-60-nife).

be investigated by evaluating the differences between the SHR-SP and WKY animals (Fig. 6A, B & C). The analysis was completed in both age groups. At the age of 40 weeks, we found no change in BMT while we saw increased fibrosis in the SP-40. The level of pericytic degeneration was basically not altered. The difference in the percentage of the individual morphological aberrations between WKY-40 and SP-40 rats did not reach significance. At 60 weeks of age, the percentage of both BMT and fibrosis remarkably increased in the SP-60-plac compared to the WKY-60-plac while the occurrence of degenerative pericytes remained similar. The graphs seen in Fig. 6D, E & F also characterize the relationship between microvascular morphological degeneration and SBP. (Here, not only the placebo experimental groups but also the treated groups, which are described in detail below, were plotted.) BMT and fibrosis showed a positive and significant correlation to SBP while the per-

Fig. 4. Average food- and water intake per animal, and body weight expressed as group mean values during the 20 weeks experimental period. A: food intake of the WKY animals. B: food intake of the SP animals. C: water intake of the WKY animals. D: water intake of the SP animals. E: body weight of the WKY groups. F: body weight of the SP groups. Significant differences in body weight based on repeated measurement ANOVA: WKY-60-plac x SP-60-plac: $F = 43.363$, $**P \leq 0.000$; WKY-60-nife x SP-60-nife: $F = 12.48$, $*P \leq 0.005$; SP-60-plac x SP-60-nimo: $F = 7.156$, $*P \leq 0.02$.

centage of microvessels with degenerative pericytes appeared to be independent of SBP.

The protective effects of nimodipine and nifedipine on cortical capillary ultrastructure can be visualized if we compare control and treated animals (Fig. 6A, B & C). In WKY,

the influence of either of the two drugs on BMT or fibrosis could not be demonstrated. However, nifedipine appeared to be able to decrease pericytic degeneration in WKY-60-nife when compared to WKY-60-plac. Thus, it seems that at physiologically optimal SBP values, nimodipine and nifed-

Fig. 5. Scanned images of original electron microscopic photographs. A: intact cerebral capillary of a WKY-40 animal. B: BMT of an SP-40 rat. C: BMT seen in the cortex of an SP-40 rat. D & E: fibrosis as it appears in SP-60-plac animals. F & G: degenerative pericytes in SP-60-plac rats. Abbreviations: e: endothelial cell, p: pericyte, *: capillary lumen, arrowheads: vascular basement membrane.

ipine do not alter the ultrastructure of the capillary basement membrane but nifedipine appears to be a drug that acts on pericytes. Treatment of the hypertensive animals with the two drugs yielded to remarkable improvement in capillary

morphology. Nimodipine decreased the incidence of BMT from 40% (SP-60-plac) to 33% (SP-60-nimo) but the compound had no significant effect either on fibrosis, or on degenerating pericytes. Nifedipine treatment lead to obser-

Fig. 6. The incidence of capillary basement membrane pathology (A, B) and pericytic degeneration (C) at the age of 60 weeks expressed in median percentages, and the correlation between systolic blood pressure and microvascular morphology (D, E, F); *: $P < 0.05$; **: $P < 0.01$.

vations similar to that of nimodipine. BMT was significantly decreased in SP-60-nife while fibrosis was unaffected. Interestingly, nifedipine improved the condition of pericytes (SP-60-nife), which finding was not seen after nimodipine treatment (SP-60-nimo).

Fig. 7C & D show the average capillary diameters calculated for each experimental group. The values ranged between $9.955 \mu\text{m}$ (SP-60-nimo) and $10.91 \mu\text{m}$ (WKY-60-nife). The microvascular lumen was consistently narrower

in the hypertensive animals than in the normotensive controls, which neither nimodipine nor nifedipine treatment could noticeably influence. When capillary diameters were related to SBP of the individual rats, a negative correlation was established. The density of capillaries considerably varied but when the three WKY-60 groups and the three SP-60 groups were combined, an increased number of capillary profiles was detected in the given cortical area in the hypertensive animals. The correlation of capillary number

Fig. 7. Capillary diameter values (C, *: $P < 0.05$; **: $P < 0.01$) and capillary density (D, $P < 0.05$).

per area unit with SBP did not reveal clear correspondence with an elevated SBP.

4. Discussion

Here we have assessed ultrastructural damage of rat cerebrocortical capillaries under chronic hypertensive conditions. The morphological abnormalities described in this study, namely fibrosis and BMT were also identified in larger brain vessels in hypertensive humans [42]. Similar microvascular abnormalities were reported previously in the cerebral cortex of aging, spontaneously hypertensive rats, which was, however, restricted to a qualitative description of capillary pathology [30]. The present study provides quantitative data, which show that BMT and collagen deposits occur considerably more frequently in aging hypertensive rats than in normotensive age-matched controls. Moreover, such vascular irregularities were not increased in 60 weeks old normotensive rats when compared to a 40

weeks old normotensive group. On the other hand microvascular BMT and fibrosis markedly increased in 60 weeks old hypertensive animals compared to 40 weeks old SHR-SP rats. Based on this finding, it may be concluded that hypertension accelerates capillary damage in the brain.

Our results also indicate that chronic nifedipine or nimodipine treatment can prevent the progression of capillary wall damage in the rat cerebral cortex. It is known that dihydropyridines decrease blood pressure by binding to L-type calcium channels on vascular smooth muscle cells and inhibit vascular contractility [1,16,28], which, in turn, gives rise to vasodilatation and reduced vascular resistance [51]. However, the two drugs have selective effects: varying doses of nifedipine have been shown to consistently decrease blood pressure while nimodipine action on tension appeared to be concentration dependent [17,18,20,33,46]. At the dose used in the present study, only nifedipine, but not nimodipine decreased SBP. Although both compounds improved the condition of cerebral capillaries in a similar fashion, only the nifedipine-mediated microvascular protec-

tion correlated with decreasing SBP while nimodipine action was independent of pressure. Thus, the processes underlying the protective effect of the two drugs on cortical capillaries must be based on more than just lowering blood pressure.

The location of L-type calcium channels in the microvascular domain, which are the principal targets of nimodipine and nifedipine, may offer further explanations for the different protective mechanisms of the two drugs. The primary building blocks of capillaries are the endothelial cells, which seem not to be endowed with L-type channels, although the occurrence of the L-type channels on the endothelium is a matter of conflicting evidence. Preparations of isolated bovine vessels or rat pulmonary artery indicated either the presence [3,37] or the absence [22] of L-type calcium channels on endothelial cells but a generally promoted concept claims the latter conclusion [1]. Accepting the lack of L-type channels on the endothelial surface, it is doubtful that nifedipine or nimodipine can directly act on the endothelium.

Nifedipine was described to block cellular calcium influx by binding to L-type calcium channels on peripheral vascular smooth muscle. By doing so, nifedipine attenuates the contractility of these vascular elements, which leads to decreased blood pressure [1,16,28]. Thus, it seems attractive to reason that vascular protection in our nifedipine treated animals was achieved by reducing SBP, which assumption would agree with the correlation found between the percentage of intact vessels and SBP. However, the finding that nimodipine treatment achieved similar microvascular protection in our paradigm without lowering SBP asks for another explanation. Since nimodipine and nifedipine were described to penetrate the brain [25,32,45,46,50] where they can block calcium influx at neuronal elements [11,44,45], the mechanisms stimulated by the drugs to preserve capillary integrity may very well lie at CNS level. Astrocytes are closely related to cerebral microvessels and participate in the formation and maintenance of the BBB. The astrocytic endfeet embrace the vascular basement membrane and were described to be able to regulate the intracerebral vascular tone and cerebral blood flow indicated by the expression of serotonergic and cholinergic receptors on the perivascular endfeet [4,5,12,26,36,41]. Furthermore, astrocytes were also reported to secrete basement membrane components [54].

The calcium homeostasis of brain cells including astrocytes can be compromised in ischemia. A mild, chronic ischemic condition in hypertension may emerge from a reduced cerebral blood flow (CBF) reported in hypertensive patients as well as hypertensive rats [19,27,38,39]. The here-used dihydropyridines nimodipine and nifedipine were shown to increase CBF in SHR rats and rabbits [40,48,49, 53] while clinical trials with nimodipine demonstrated that the drug improved CBF without having an influence on systemic blood pressure [17,18,46]. These latter results are

intriguing in the light of our data which show that nimodipine prevented microvascular breakdown without affecting SBP.

Cerebral hypoperfusion, thus also occurring in hypertension, is known to impose a mild ischemic condition. The metabolic effects of reduced cerebral blood flow were studied in animal models of chronic bilateral carotid artery occlusion. Using this hypoperfusion model, de la Torre and co-workers reported, for example, a decreased cytochrome oxidase activity in neuronal mitochondria suggesting a mild ischemic condition in chronic cerebral hypoperfusion [10], which implies an increased intracellular calcium concentration that can impose harmful effects on mitochondrial function. Furthermore, the elevated calcium concentration is known to activate detrimental metabolic cascades and is accompanied by free radical generation [47]. The calcium-generated synthesis and accumulation of the easily translocating NO and its toxic products [2,34] can attack the integrity of the BBB: it may cause astrocytic dysfunction [43] and increased endothelial permeability [24,31,52]. The here presented microvascular pathology in SHR-SP animals can be associated with such a chain of events. Nimodipine treatment can supposedly prevent the intracellular calcium accumulation, thus moderating free radical production [55] and consequent cerebral capillary injury.

The possibility that a decreased CBF-induced, mild but persistent ischemic condition would contribute to the capillary damage seen here could be reflected by changes in capillary density since cerebral ischemia has been shown to promote microvascular proliferation. For example, hypoxia has been demonstrated to lead to the enlargement and proliferation of the microvascular endothelial cells and a consequent increase of cerebral capillary density [21]. Capillary density was also reported to correlate with the metabolic rate for glucose [23]. Our data indicate a hypertension-related decrease in capillary diameter and an increase of capillary density in the cortical region investigated. These observations can raise the following assumptions. Considering that the endothelial cells and pericytes presumably possess contractile properties [29], a generally narrower capillary lumen may reflect vasoconstriction typically associated with hypertension. The decreased capillary diameter can be responsible for a lower CBF, which would give rise to a mild, chronic ischemia represented by the elevated capillary density. Of course, such hypothesis can only be fully appreciated if the detection of typical indicators of ischemia (reduced glucose utilization and oxygen consumption) give support to the vascular observations. Nevertheless, a persisting but moderate ischemic condition may well be causal to the here-described capillary damage, which could be prevented by dihydropyridines.

In summary, it has been shown in this study that hypertension can accelerate age-related, ultrastructural capillary malformations. Microvascular degeneration appears in the form of vascular basement membrane pathology, such as BMT and fibrosis. Chronic treatment with dihydropyridine

drugs, in this case with nimodipine and nifedipine can prevent the progression of microvascular damage by reducing the ratio of cerebral capillaries featuring BMT. Thus, in addition to the well-described beneficial effects of dihydropyridines on neuronal networks, nimodipine and nifedipine have been shown to improve the condition of cerebral capillaries in chronic hypertension. The underlying mechanism of the process may be related to an increase in CBF, which leads to an improved neural regulation of cerebrovascular function. The precise chain of events, however, is still to be elucidated.

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