On the Activation of Calcium-Dependent Proteolysis in Brain Neurons of Spontaneously Hypertensive Rats (SHR Strain)

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Females of spontaneously hypertensive (SHR strain) and normotensive rats (WKY strain and Wistar) received drinking water with normal (80 mg/liter) or reduced concentration of Ca^{2+} (8 mg/liter). Activity of calcium-dependent calpain protease in neurons did not differ in 18-day-old rat pups born and suckled by these animals. Our results are consistent with published data on normal metabolism of SHR rats up to the age of 30 days.

Key Words: *hypertension; spontaneously hypertensive rats; calcium paradox; calcium-dependent calpain protease*

Spontaneously hypertensive rats (SHR strain) serve as a convenient model for evaluation of the mechanisms of blood pressure (BP) elevation. The hereditary characteristic of these animals is elevated Ca^{2+} concentration in smooth muscle cells (*e.g.*, in cells of blood vessel walls). It results in the contraction of smooth muscles, vasoconstriction and, therefore, increase in BP [7,11]. BP in SHR rats can be reduced by feeding a diet with a 3-fold higher content of Ca^{2+}. By contrast, the decrease in dietary Ca^{2+} is followed by a progressive increase in cytoplasmic Ca^{2+} concentration [3,10]. The mechanisms of this phenomenon remain unclear. Some authors believe that dihydroxylated vitamin D ($1,25\text{-}(OH)_2\text{-D}$) plays the key role in the regulation of Ca^{2+} influx into smooth muscle cells [14]. Its synthesis decreases with increasing Ca^{2+} concentration in the intercellular space. Ca^{2+} influx into cells decreases under these conditions. On the other hand, feeding a low-calcium diet is accompanied by enhanced secretion of parathyroid hormone, which contributes to Ca^{2+} outflow from the bones, accumulation of Ca^{2+} in soft tissues, and increase in intracellular Ca^{2+} concentration [4].

Previous studies showed that SHR rats have some behavioral disturbances [6]. It can be suggested that brain cells in these rats (*e.g.*, neurons) differ in hereditary characteristics. Ca^{2+} concentration is elevated in the cytoplasm of neurons and smooth muscle cells. These data are consistent with increased influx of Ca^{2+} into synaptosomes (isolated axons of neurons) of SHR rats as compared to that in animals of other strains [5]. The increase in cytoplasmic Ca^{2+} concentration causes an imbalance between calcium-dependent processes in neurons, which affects the formation of neuronal networks and behavior of animals. This concept is confirmed by published data on increased content of cytoplasmic Ca^{2+} in brain cells of spontaneously hypertensive rats [2].

The elevation of cytoplasmic Ca^{2+} concentration is accompanied by changes in cellular metabolism (*e.g.*, increase in activity of calcium-dependent calpain protease). This protease exists in the following two forms: μ-calpain and m-calpain. They are activated in the extracellular systems with micromolar and millimolar concentrations of Ca^{2+} in the medium, respectively. Previous studies showed that neuronal protein GAP-43

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serves as a m-calpain substrate, which splits GAP-43 (the only site near Ser41) [13]. The C-terminal fragment GAP-43-3 (20 kDa) is identified in immunoblots after treatment with polyclonal antibodies to GAP-43. The elevation of GAP-43-3 in brain protein samples (or decrease in the number of GAP-43 molecules) reflect hyperactivity of m-calpain and, therefore, significant increase in cytoplasmic Ca^{2+} concentration in neurons. Protein spectrin can be used as a fine marker of elevation of cytoplasmic Ca^{2+} concentration in neurons. Spectrin serves as a μ-calpain substrate. *In vitro* activation of μ-calpain occurs at a lower concentration of Ca^{2+} in the medium (as compared to that for m-calpain) [15]. The same regularities are probably typical of living cells.

This work was designed to evaluate whether a diet with the reduced or normal content of Ca^{2+} is associated with increased activity of calpain and cytoplasmic Ca^{2+} concentration in neurons of SHR rats. The analyzed situation (experiments on newborn animals and only drinking water is Ca^{2+}-deficient) is of considerable importance for applied dietetics.

MATERIALS AND METHODS

Experiments were performed on 18-day-old rat pups from SHR, WKY (normotensive control), and Wistar rats. The mothers were divided into 2 groups. Both groups received a standard dry feed to meet the daily calcium requirements for 3 months before pregnancy and then during period pregnancy and nursing. The mineral composition of drinking water was different for animals of groups 1 (normal content of Ca^{2+}, 80 mg/liter) and 2 (low content of Ca^{2+}, 8 mg/liter).

The specimens (mouse brain) were kindly provided by N. Z. Klyueva and E. I. Petrova (I. P. Pavlov Institute of Physiology, Russian Academy of Sciences). The synaptosomal fraction was isolated as described previously [13]. Synaptosomes were lysed with aqueous solution of sodium dodecyl sulfate (2%) at 100°C for 2 min to solubilize the synaptosomal proteins. Proteins were separated by electrophoresis 12% PAAG in the presence of sodium dodecyl sulfate (Laemmli method) [8]. The separated synaptosomal proteins were placed on a nitrocellulose membrane (immunoblotting technique). Immunochemical identification of proteins was conducted with polyclonal antibodies of the corresponding specificity (Chemicon).

RESULTS

The content of total proteins (GAP-43, αII-spectrin, and BASP1) and their fragments was measured in axon terminals (synaptosomes) of brain neurons from SHR, WKY, and Wistar rat pups, whose mothers received drinking water with various concentrations of Ca^{2+}. The loss of proteins during isolation could affect quantitative ratio between these proteins. Hence, portions of lysed synaptosomes were put on the gel for electrophoretic separation of study proteins. Each portion contained the same amount of total proteins. Several independent series were performed. The results of typical experiments are presented as an immunoblot of proteins. Previous studies showed that neurons of Wistar rats contain not only full-length GAP-43 protein, but also the m-calpain-produced fragment GAP-43-3 [13]. Its content is high in the brain of late embryos and newborn rats [9]. The content of GAP-43-3 in synaptosomes did not differ in 18-day-old pups of Wistar, WKY, and SHR rats (Fig. 1). The decrease in Ca^{2+} concentration in drinking water had no effect on the content of the intact protein GAP-43 or GAP-43-3 fragment (shown for SHR rats; Fig. 1).

The protein αII-spectrin serves as a substrate for μ-calpain. Activation of μ-calpain occurs at a lower concentration of Ca^{2+} in the medium (as compared to that for m-calpain). Immunoblot assay showed that the degree of spectrin fragmentation in neurons from 18-day-old SHR rat pups is not increased after consumption of drinking water with normal or reduced concentrations of Ca^{2+} (Fig. 1, shown for SHR rats). Similar results were obtained in studying the animals of all strains.

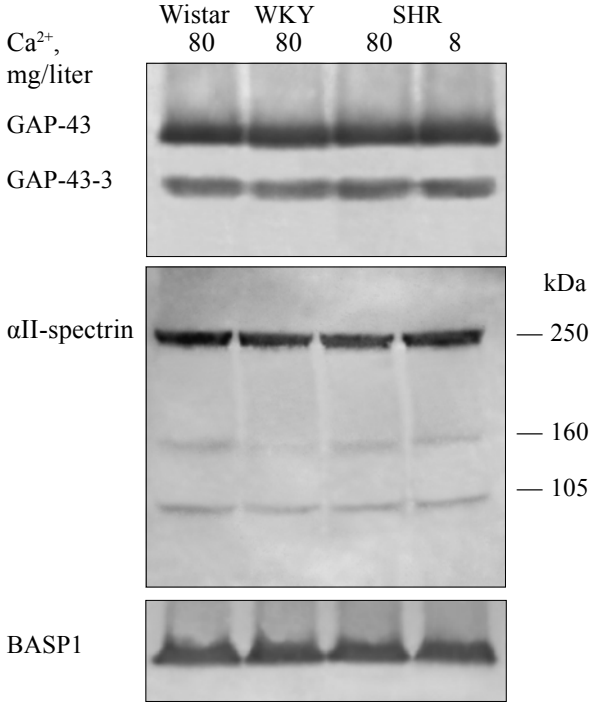

Fig. 1. Immunoblots of total proteins and their fragments in the isolated axon terminals of neurons (synaptosomes) from 18-day-old rat pups of WKY, SHR, and Wistar strains after consumption of drinking water with a normal or reduced concentration of Ca^{2+}.

BASP1 content was practically the same in 18-day-old rats of various strains. The amount of this protein did not depend on the presence of normal or reduced concentration of Ca^{2+} in drinking water (Fig. 1). BASP1 fragmentation was not observed under various experimental conditions.

Therefore, no increase in calpain activity was revealed. It cannot be expected that cytoplasmic Ca^{2+} concentration is increased in SHR rat pups. This study failed to confirm the hypothesis that cytoplasmic Ca^{2+} concentration in brain cells of SHR rat pups is elevated after consumption of a low-calcium diet (drinking water) by mothers. At the first glance, our findings seem to contradict the results of previous experiments. It was found that cytoplasmic Ca^{2+} concentration is high in SHR rats. Moreover, cytoplasmic Ca^{2+} concentration was shown to increase progressively with a decrease in the amount of dietary Ca^{2+} [14]. It should be emphasized that these experiments were performed on adult animals (older than 8 weeks). Expression of the protein factor, which induces the increase in cytoplasmic Ca^{2+} concentration, was observed in SHR rats aged more than 4 weeks [1]. Various authors reported that cytoplasmic Ca^{2+} concentration and BP in 4-week-old SHR rats do not differ from normal [11,12]. The same features should be observed in rat pups younger than 3 weeks (used in our experiments). Moreover, experimental animals (mothers of rat pups) received a standard diet to meet the daily calcium requirements and only drinking water contained the reduced amount of Ca^{2+}. Therefore, these animals were exposed to mild Ca^{2+} deficiency. These facts can explain the absence of significant changes (*e.g.*, activation of calpain).

The result of our experiments can be considered as "negative", which agrees well with published data. We did not plan to publish these findings. The experimental work was still continued. However, we decided to prepare this manuscript after publication of N. Z. Klyueva *et al*. [1]. It contains only some data that were obtained at the early stage of this study (Figs. 2 and 3 [1]). Moreover, the designations of SHR and WKY strains in Fig. 3 of the cited article [1] were confused. The authors concluded that Ca^{2+} deficiency in drinking water is accompanied by activation of calpain and, therefore, increase in cytoplasmic Ca^{2+} concentration in neurons of 18-day-old SHR rats. These data were not reproduced in subsequent experiments repeated many times (Fig. 1) and, therefore, should be considered as erroneous.

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