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Changes of Calbindin-D28k Expression Levels After Transient Ischemic Damage in Rat Cerebellar Purkinje Cells

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Cerebellar Purkinje cells are selectively vulnerable to ischemia, although the reasons for this are not known. Moreover, an intracellular Ca^{2+} overload induced by excitotoxicity (toxic glutamate receptor activation) is considered to be a key mediator of central neuronal loss consequent to ischemic damage. Calbindin-D28k is an intracellular calcium binding protein that is expressed in nearly all Purkinje cells of the rat cerebellum. Its major role is presumed to be associated with intracellular Ca^{2+} buffering. In the present work, In-Situ Hybridization and Western blot methods were used to investigate the changes of calbindin-D28k and its gene expression levels in the rat cerebellum at various times after transient global ischemia. Both level of calbindin-D28k and its mRNA expression level in the cerebellum decreased after ischemic insult, whereas the number of cerebellar Purkinje cells was unaltered after ischemia. In the light of our finding of lower levels of calbindin-D28k and its mRNA in the cerebellum, altered intracellular calcium buffering capacity in the cerebellar Purkinje cell might be presumed. It is believed that this may lead to calcium-mediated cytotoxic events after ischemic insults in cerebellum.

Key words : Calbindin-D28k, Ischemia, Cerebellum, Purkinje cell

Introduction

Calbindin-D28k is a calcium-binding protein of the E-F hand family (Lomri *et al*. 1989, Minghetti *et al*. 1988), and is widely expressed throughout the rat brain (Celio 1990), including the Purkinje cells of the cerebellum (Kadowaki *et al*. 1993). Among the many calcium-binding proteins in the nervous system, calbindin-d28k is particularly notable in terms of its abundance and distribution specificity. As a result, many neuroanatomical and developmental studies have used calbindin-D28k as a cellular marker (Andressen *et al*. 1993).

Calbindin-D28k is known to have a neuroprotective role and the maintenance of the concentration of calbindin-D28k in certain neurons may be critical for cell survival. Several research groups have found altered expression of calcium binding proteins, including calbindin-D28k in normal aging (Wu *et al*. 1997, Kishimoto *et al*. 1998) and in association with acute insults, such as stroke and epileptic seizures, and also in chronic neurodegenerative disorders, such as Alzheimer's, Huntington's, Parkinson's and Pick's diseases (Heizmann and Braun 1992). In view of the importance of calcium for proper neuronal maintenance and function and the proposed physiological role of calbindin-D28k with respect to intracellular calcium (Baimbridge *et al*. 1992, Heizmann and Hunziker

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1991, McMahon *et al.* 1998), it is possible that abnormalities in calcium homeostasis in various neurotoxic conditions are closely linked to changes in the intracellular calbindin-D28k level.

Recent studies (Diemer *et al.* 1993, Chamimoniuk and Strosznajder 1998, Martin *et al.* 2000) have indicated that neuronal cell death after ischemic insult involves perturbations in intracellular Ca^{2+} homeostasis, possibly resulting from excitotoxic activation of neuronal glutamate receptors. Since cerebellar Purkinje cells are rich in calbindin-D28k and selectively vulnerable to ischemia, although the reasons have not been discovered until now, we considered it would be useful to study the relationships between neuronal vulnerability after ischemic insult and associated changes of calbindin-D28k expression level in the cerebellum.

Thus, the goal of this study was to provide insight into the possible contribution that changes of calbindin-D28k expression levels make degeneration in cerebellar Purkinje cells after ischemic insult. In the present work, histological examination with the In-Situ Hybridization techniques demonstrated the effect of ischemia-reperfusion insult on calbindin-D28k mRNA expression levels at the cellular level. Western Blot with polyclonal anti calbindin-D28k antibody was also used in this study to investigate changes of calbindin-D28k levels in the cerebellum after ischemic insult.

Materials and Methods

1. Animal experiment

In this study, we used a two-vessel occlusion (common carotid artery) model of transient global ischemic insult. 10 young adult (2~3 months) male F344 rats were used in each experimental group (sham-operation, postischemic days 1, 2, and 7). Animals were anesthetized with diethyl ether and both common carotid arteries were carefully dissected and clipped for 15 minutes with aneurysm clips (AESCULAP™). After surgery, anesthesia was discontinued and the rats were allowed free access to food and water until they were sacrificed on either reperfusion days 1, 2 or 7. Sham-operated rats were subjected to the same procedure, except that the aneurysm clip was not applied. Animal care and the experimentation complied with the NIH guide for the care and use of laboratory animals.

2. In-Situ Hybridization

Frozen coronal sections (10 μm) of rat cerebellum were thaw-mounted onto gelatin-coated slides, fixed in 4% paraformaldehyde (pH 7.0) for 10 min., dehydrated in graded alcohols, treated with chloroform for 4 min., and rehydrated. pGEM-T (Promega™) vector inserted rat calbindin-D28k gene coding region (786 bp) was labeled with DIG (Digoxigenin)-dUTP (alkali-labile) in the polymerase chain reaction (PCR) using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim™). Slides were incubated in a humidified chamber at 42°C for 18 hr with 75 μl of hybridization solution per slide (25% formamide, 60% Hybrizol I (Oncor™), and 15% DEPC D.W.). After hybridization, the slides were incubated in SSC buffer containing 50% formamide for 30 min at 50°C, and then incubated with alkaline phosphatase conjugated Anti-DIG (Boehringer Mannheim™) for 45 min at 37°C. Finally the colorimetric reaction was performed with NBT/BCIP solution (Boehringer Mannheim™) for 3~4 hrs at 37°C.

3. Western blot

The cerebellum was extracted from rat whole brain and homogenized with lysis buffer (10% PBS, 1% Triton X-100™), 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF). Samples so obtained were applied to 15% SDS-polyacrylamide gel electrophoresis and

electrophoretically transferred to nitrocellulose membranes using semi-dry transfer units (TE70, Hoefer TM). Calbindin-D28k was detected with polyclonal anti calbindin-D28k (Oncogene™) and enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech™). Finally, the results were analyzed using an image-analysis system (Vilber Lourmat™).

4. Quantification of the number of cerebellar Purkinje cells

The cerebellum was sectioned to 40 μm and Nissl stained with cresyl violet to count the number of cerebellar Purkinje cells. Five contiguous sections of cerebellum were selected from each group and all Purkinje cells were counted in areas including, the simple lobule, ansiform lobule and vermis. Finally analysis of variance (ANOVA) testing ($p < 0.05$) was performed to compare the significance of differences among the experimental groups.

Results

When examined by In-Situ hybridization, calbindin-D28k mRNA expression was found to be localized almost exclusively in the Purkinje cell layers in the cerebellar cortex of the sham operated control rat. After ischemic insult, moderate edematous changes were observed in hippocampal and striatal portions of cerebral hemispheres (data not shown). These changes were more prominent during the late reperfusion time and were similar bilaterally, but there were no particular pathologic changes in the cerebellum on gross examination. No statistically significant differences in the number of Nissl stained cerebellar Purkinje cells were found in the experimental groups by ANOVA test, which might suggest that changes of calbindin-D28k and mRNA expression levels are relatively early events in the ischemia induced apoptosis or necrotic degeneration of Purkinje cells (Fig. 1).

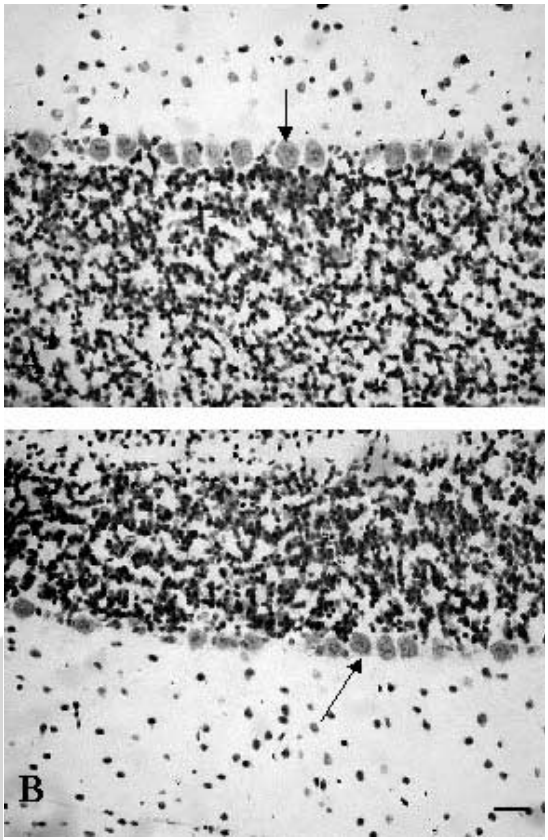

Fig. 1. Photomicrographs of Nissl stained (Cresyl Violet staining) lobule of cerebellar cortex. (A) 1 day after reperfusion. (B) 7 days after reperfusion. Note no significant numerical change between two groups Scale bar = 50 μm, Arrows = Nissl stained cerebellar Purkinje cells

After 15 minutes of transient ischemic insult, calbindin-D28k and its mRNA expression levels decreased during the reperfusion periods (day 1, 2 and 7). In-Situ hybridization showed depleted calbindin-D28k mRNA expression levels in almost all cerebellar Purkinje cells after ischemic insult. In the sham operated control rat, Purkinje cells were stained strongly with NBT-BCIP in almost all cerebellar cortical areas, including the simple lobule, ansiform lobule

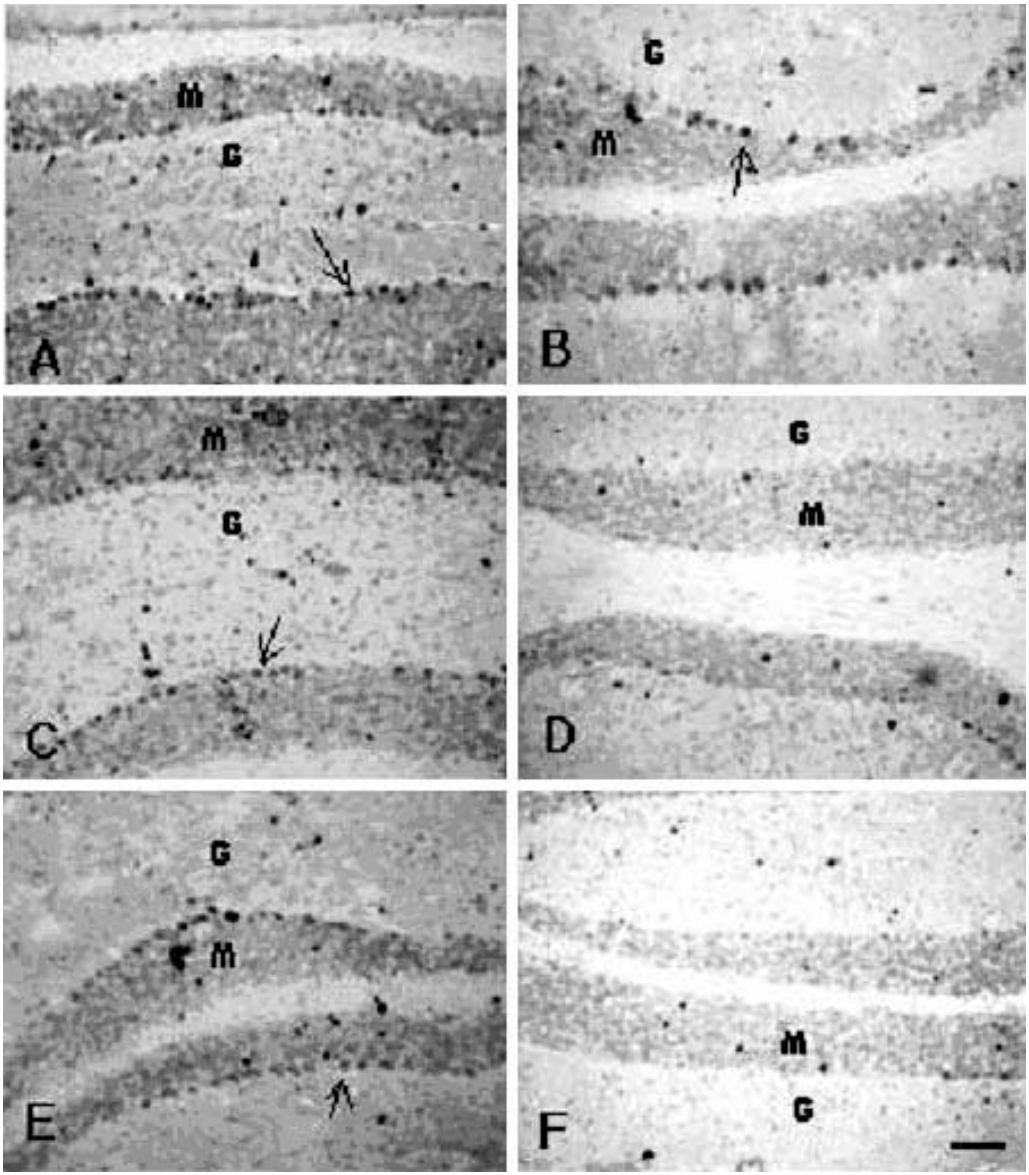

Fig. 2. In-Situ hybridization of calbindin-D28k mRNA expressing Purkinje cells (arrows) in the lobule of cerebellar cortex. Note the almost depleted calbindin-D28k mRNA expression level (D, F) compared to the sham-operated control after 2 and 7 days of reperfusion (C, E). A. 1 day of reperfusion (sham operated control), B. 1 day of reperfusion, C. 2 days of reperfusion (sham operated control), D. 2 days of reperfusion, E. 7 days of reperfusion (sham operated control), F. 7 days of reperfusion, M: molecular layer, G: granular layer, Scale bar = 100 μm

and vermis. Whereas, there were no particular changes during the early reperfusion stage (1 day after ischemic insult), calbindin-D28k mRNA expression was almost absent after 2 and 7 days of reperfusion (Fig.

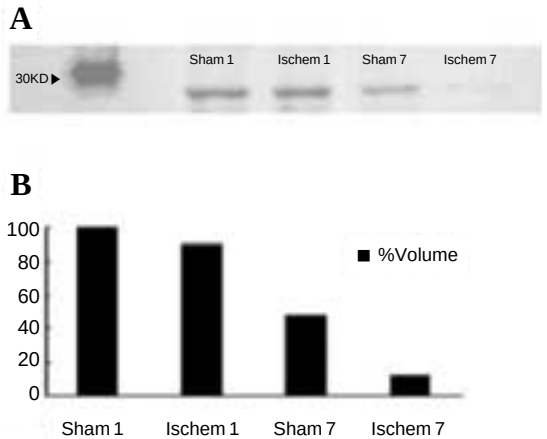

Fig. 3. Western blot analysis of calbindin-D28k protein expression in the cerebellar cortex (15 g of protein/lane) from the sham control and ischemic rats after 1 day (Sham1, Ischem1) and 7 days (Sham7, Ischem7) of reperfusion. The calbindin-D28k level in ischemic rats decreased significantly after 7 days of reperfusion, whereas no changes were observed between the sham control and ischemic rats after 1 day of reperfusion.

2).

Western blot analysis using polyclonal anti calbindin-D28k antibody was used to examine the amount of calbindin-d28k in the sham operated and ischemic model rats. 28-kDa calbindin protein was observed and found to decrease during periods of reperfusion. After 7 days of reperfusion, we found that the calbindin-D28k level decreased prominently in ischemia-induced subjects compared to sham operated subjects. Although the amount of calbindin-D28k in the sham operated group decreased, as did its level in the ischemia group, the degree of reduction was markedly different (Fig. 3).

Discussion

This study indicates that transient ischemic insult induces changes in both the expression level of calbindin-D28k and its mRNA in cerebellum. Although the number of Purkinje cells was unaltered after ischemic insult, ischemic insult influenced the changes of calbindin-D28k gene expression level during the ischemia-reperfusion periods. Considering the relatively short post-ischemic periods (day 1, 2, and 7) involved in this experiment, those changes seemed to be occurred before the loss of Purkinje cells caused by delayed degeneration. Although the Purkinje cells were known to be vulnerable to ischemic insult, as mentioned in introduction, a recent report indicated delayed degeneration of Purkinje cells in transient global incomplete cerebral ischemia (Martin *et al*. 2000).

Our findings provide the first evidence of changes in the calbindin-D28k expression levels after ischemic insult in cerebellar Purkinje cells, in which calbindin-D28k is known to be the predominant cytosolic protein. It has been suggested that perturbations in intracellular Ca^{2+} homeostasis, induced by toxic glutamate receptor activation, is a key mediator of central neuronal loss consequent to hypoxic-ischemic insults. Therefore, it seems possible that a reduction of calbindin-D28k gene expression depletes the neuron's ability to buffer cytosolic free calcium, rendering it susceptible to the initiation of calcium-mediated irreversible cytotoxic events. Thus, as mentioned in introduction, it is possible that an abnormality in cal-cium homeostasis in an ischemia-reperfusion injury to the brain is closely linked to changes in the calbindin-D28k gene expression.

With regard to ischemic insult to the brain and its relation with calbindin-D28k, the majority of studies to date have been limited to the hippocampal region. Ischemic insult in the adult rat is associated with a characteristic pattern of hippocampal cell vulnerability, with dentate hilar neurons and CA1 (cornu ammonis area 1) pyramidal cells being the most vulnerable. Calbindin-D28k immunoreactive granule cells were reported to be selectively resistant to ischemic insult in the dentate gyrus of the developing rat

(Goodman *et al*. 1993), and vulnerability seemed to be inversely correlated with the calbindin-D28k contents of the hippocampal neurons (Freund *et al*. 1990, Rami *et al*. 1992). However, another theory suggests that a mobile cellular calcium buffer could promote neuronal injury. In a study using calbindin-D28k knockout mice (Klapstein *et al*. 1998), it was suggested that a calbindin-D28k deficient status is more favorable against ischemic insult in spite of its cytoplasmic calcium buffering properties. It seems possible that other calcium-binding proteins may have been up-regulated or other compensatory mechanisms initiated before the time of insult. Therefore, further studies upon various aspects of this issue are required to clarify whether the calcium buffering property of calbindin-D28k is really neuroprotective. Nevertheless, it is generally accepted that the regulation of intracellular Ca^{2+} concentration by calcium-binding protein is a critical aspect of neuronal survival (Orrenius and Nicotera 1994), and that there exists strong relationships between various neurodegenerative diseases and calcium-binding proteins, including calbindin-D28k. Although we didn't measured the intracellular calcium concentration elucidate the mechanisms involved in lowering the calbindin-D28k gene expression in this study, we observed almost completely depleted calbindin-D28k mRNA expression in the Purkinje cells and an associated significant decrease in its level in the cerebellum. This situation may lead to a failure of calcium buffering or intraneuronal calcium homeostasis, which contributes to excitotoxicity-mediated events during the ischemia-reperfusion period. Since Purkinje cells are known to be vulnerable to ischemic insult, and calbindin-D28k is a predominant protein in the Purkinje cell, we consider that cerebellar Purkinje cells, along with calbindin-D28k, would be a very useful subject for studying the pathophysiology of ischemia-reperfusion or other calcium mediated toxic injuries.

We are now further investigating the role of calbindin-D28k using an in-vitro calbindin-D28k overexpression system, and direct measurements of intracellular Ca^{2+} will be made for the next step. Supplementing the Ca^{2+}-binding capacity of vulnerable cells via gene transfer of calbindin-D28k may reduce neuronal damage by interrupting the cascade of Ca^{2+}-induced neurodegenerative events (Phillips *et al*. 1999), since minute changes in intracellular Ca^{2+} have profound effects on neuronal cell functions. Future studies should focus on the physiological roles of these proteins in the CNS, using calcium binding protein gene transfected neuronal cell lines and the transgenic modeling of neurodegenerative events. The outcome of these results may also be relevant to the medication developed to correct the hyperactivity of intracellular Ca^{2+}.

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허혈 재관류에 의한 흰쥐 소뇌 Purkinje 세포의 칼슘결합단백(calbindin)의 변화

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소뇌에 존재하는 Purkinje 세포는 아직 그 이유는 자세히 모르나 뇌허혈(brain ischemia)에 매우 민감한 것으로 알려져 있다. 또한 흥분독성(excitotoxicity; toxic glutamate receptor activation)에 의해 유발된 세포내 Ca^{2+}의 과도한 증가는 뇌허혈에 의한 중추신경세포의 손실에 있어서 중심적인 매개체 역할을 하게 된다. Calbindin-D28k는 흰쥐 소뇌의 거의 모든 Purkinje 세포에서 발현되는 세포내 칼슘결합단백질로서, 주된 기능은 세포내의 Ca^{2+}에 대한 완충작용인 것으로 여겨지고 있다. 본 연구에서는 흰쥐 뇌의 모든 부위에 일시적 허혈을 유발시킨 뒤 재관류 시간별로 소뇌에서의 calbindin-D28k의 발현변화를 In-Situ Hybridization과 Western Blot을 통하여 알아보았다. 15분간 일시적 허혈을 유발시킨 다음 재관류 일주일 후에도 소뇌 Purkinje 세포의 숫적 변화는 없었던 반면 calbindin-D28k의 발현 변화는 재관류 초기에 현저히 변하는 것이 확인되었다.

따라서 뇌허혈은 소뇌 Purkinje 세포의 세포내 칼슘완충능력의 변화를 야기시키게 되고 이것이 칼슘 매개성 세포독성을 유발시킬 것으로 여겨지며, 본 연구에서 밝혀진 결과는 향후 뇌허혈에 의해 유발되는 신경세포의 병태생리를 이해하는데 필요한 중요한 자료를 제공할 것으로 생각한다.

찾아보기 낱말 : Calbindin-D28k, 뇌허혈, 소뇌, Purkinje cell