The effects of the Na^+/Ca^++ exchange blocker on osmotic blood-brain barrier disruption

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【学位論文題目】

The effects of the Na^+/Ca^{++} exchange blocker on osmotic blood-brain barrier disruption.

（血液脳関門の浸透圧性破綻に対する
Na^+/Ca^{++} 交換阻害剤の効果）

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Introduction

The Blood-brain barrier (BBB) is an endothelial permeability barrier present in capillaries and selectively limits the influx and efflux of a wide variety of solutes and substances between blood and brain. The BBB is a dynamic and regulated structure but its barrier function can be disrupted physiologically by means of hyperosmotic stress. The intra-arterial infusion of mannitol has been theorized to cause vasodilatation and osmotic shrinkage of endothelial cells, and consequent separation of tight junctions of the BBB. Osmotic disruption of the BBB by mannitol has been reported in both animals and humans but the time course in the early phase of disruption could not be accurately identified due to indistinct time resolution. In the study presented here, we first examined the rapid changes in cerebrovascular permeability in rats after BBB disruption by intra-arterial infusion of mannitol. We then demonstrated that the specific Na^+/Ca^{++} exchange blocker (KB-R7943) prolongs the barrier disruption induced by osmotic stress. This is the first study to demonstrate that BBB closure can be manipulated *in vivo*.

Materials and Methods

The experiments were conducted according to the Guidelines for Animal Experiments of the Kobe University School of Medicine. Adult male Sprague-Dawley (SD) rats weighing 300- 350g were used for this study. The rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). Retrograde cannulation with a 10-cm polyethylene catheter into the right external carotid artery was performed under heparinization.

In the control group, osmotic BBB disruption was attained by intra-arterial infusion of mannitol (n = 27), and in the treatment group, osmotic disruption was achieved in conjunction with intravenous (iv) injection of the Na^+/Ca^{++} exchange blocker (KB-R7943) prior to the infusion of mannitol (n = 15). For the control group, the 27 rats were infused with perfusate containing [^{14}C]-sucrose to measure cerebrovascular permeability at 5 minutes (n = 7), 15 minutes (n = 8), 20 minutes (n = 4), 30 minutes (n = 4), and 60 minutes (n =4) after the infusion of mannitol. In the treatment group, 15 rats were injected with the Na^+/Ca^{++} exchange blocker (iv, 3 mg/kg) and then infused with perfusate containing [^{14}C]-sucrose to measure cerebrovascular permeability at 5 minutes (n = 5), 30 minutes (n = 5), and 60 minutes (n = 5) after the infusion of mannitol.

In addition, rats were infused with only perfusate to determine the BBB permeability for [^{14}C]-sucrose without osmotic disruption (n =7). A histopathological study was performed to evaluate the neuronal damage following BBB disruption by the Na^+/Ca^{++} exchange blocker and mannitol (n =3).

Hypertonic agent: Mannitol was chosen as a hypertonic agent to open the BBB osmotically. *Perfusate:* To measure cerebrovascular permeability, [^{14}C]-sucrose (0.5 μCi/ml) was added to the perfusion fluid (PBS, pH- 7.4) and equilibrate prior to infusion.

In every rat, the proximal portion of the right common carotid artery (CCA) was occluded with an aneurysm clip immediately prior to the infusion of mannitol or perfusate. Mannitol was infused into the right external carotid artery at a rate of 8.3×10^{-2} ml/sec for 30 seconds with an infusion pump. Immediately after the infusion of mannitol, the clip was released and to allow for systemic blood flow to the right carotid system. To measure the cerebrovascular permeability, the right CCA was clipped again and perfusate containing [^{14}C]-sucrose was infused for 60 seconds, after which the intravascular radioisotopes were washed out by infusion of normal saline for 30 seconds and finally the rat was sacrificed by decapitation. Immediately after the brain had been removed, it was placed on a filter paper moistened with normal saline. The arachnoid membrane and meningeal vessels were removed carefully to avoid injury to the cortex. Two regions, the ipsilateral frontal and parietal cortex, were dissected and placed in vials to be weighed. The samples were then digested over night at 40°C in 1 ml of tissue solbidizer. Finally, 10 ml of scintillation cocktail for scintillation counting and 70 μl of glacial acetic acid to eliminate chemiluminescence were added. Counting was performed with a liquid scintillation counter.

The cerebrovascular permeability area product, PA (s^{-1}), was calculated as brain [^{14}C] (dpm· g^{-1})/perfusate [^{14}C] (dpm· ml^{-1})/sec. A two-sample Student's t test was employed for comparing two means with $P < 0.05$ set as the level of significance. Data were expressed as mean ± standard error.

Results

Time course of cerebrovascular permeability, PA(s^{-1}), after osmotic disruption: Without osmotic disruption, the PA(s^{-1}) was $(6.3 \pm 0.8) \times 10^{-5}$ and $(9.8 \pm 1.9) \times 10^{-5}$ for the frontal and parietal regions. Five minutes after the infusion of mannitol, the BBB was markedly disrupted. The PA(s^{-1}) peaks for [^{14}C]-sucrose were $(34.9 \pm 9.5) \times 10^{-5}$ for the frontal and $(56 \pm 14.6) \times 10^{-5}$ for the parietal region, and these peaks were 5.5 - 5.7 times higher than the PA(s^{-1}) measured without osmotic disruption. Immediately after osmotic disruption, the process of BBB closure started and the absolute value for PA(s^{-1}) progressively declined. By 30 minutes, PA(s^{-1}) had decreased significantly but not returned to the pre-infusion level. The effect of osmotic barrier disruption had been reversed completely by one hour and the value for PA(s^{-1}) had returned to the pre-infusion level.

Effect of Na^+/Ca^{++} exchange blocker on PA(s^{-1}) changes after osmotic disruption of BBB: There was no significant difference in PA(s^{-1}) after five minutes between the rats

treated with or without the Na^+/Ca^{++} exchange blocker. This indicates that the Na^+/Ca^{++} exchange blocker does not affect the degree of BBB disruption itself.

Thirty minutes after the infusion of mannitol, the increase in PA(s^{-1}) was preserved in the rats treated with the Na^+/Ca^{++} exchange blocker, which was significantly higher than the PA(s^{-1}) in rats without the blocker. These findings indicate that the Na^+/Ca^{++} exchange blocker prolongs the duration of BBB disruption. Although barrier closure tended to occur 60 minutes after infusion of mannitol, the PA(s^{-1}) for [^{14}C]-sucrose had not returned to the pre-infusion level. Sixty minutes after osmotic disruption, the rats treated with the Na^+/Ca^{++} exchange blocker showed 1.7 - 2.2 times higher PA(s^{-1}) values than those without the blocker.

Histopathological evaluation of viable neurons: On the non-perfused side, the number of viable neurons was 61.8 ± 1.8 over a 250 μm^2 area in the frontoparietal cortex compared with 62.5 ± 2.0 in the perfused frontoparietal cortex. With regard to the number of viable neurons in the frontoparietal cortex, there was no significant difference between the perfused and non-perfused side of the brain.

Discussion

Time-dependent changes in the cerebrovascular permeability after osmotic BBB disruption could be a model of reversible tight junctional opening. Hyperosmotic stress can be exerted up to 30 seconds and more than 30 seconds of infusion was found to have no incremental effect on BBB permeability. A few studies using animals have demonstrated reversibility of cerebrovascular permeability after osmotic BBB disruption. In rats, radioisotopes were administered intravenously and cerebrovascular permeability remained elevated in some areas of the brain even two hours after osmotic disruption. The time resolution for the intravenous injection method was estimated to be 10 minutes. On the other hand, the time resolution of the in situ brain perfusion method in our study was only 60 seconds. This improvement in the time resolution of our experiment enabled us to demonstrate the distinct time course of cerebrovascular reversibility within one hour. In addition, factors related to the systemic blood pressure and regional cerebral blood flow did not come in to play since the intra-arterial infusion was done at a constant perfusion rate and perfusion pressure after occlusion of the proximal common carotid artery. As a result, the extent of hemispheric crossover of perfusate was almost constant in every rat in our study. In rhesus monkey, the early time course of cerebrovascular reversibility following osmotic disruption has been elucidated using positron emission tomography. This is the first study to demonstrate the precise time course of cerebrovascular reversibility with the earliest BBB disruption occurring five minutes after the infusion of mannitol in rats.

The phenomenon of reversion of opening of the BBB consists of two separate processes, the disruption of barrier integrity and its recovery. To date, the mechanism of barrier closure after osmotic disruption, that is, the mechanism of reassembly of the tight junction, has not been identified yet. To our knowledge, ours is the *in vivo* first study to demonstrate that barrier closure can be delayed and that the duration of BBB disruption can be prolonged by pharmacological manipulation. It was proposed that vasodilatation and shrinkage of endothelial cells after hypertonic infusion may widen the interendothelial tight junctions. More recent studies have suggested the possibility that Ca^{++} induced contraction of the endothelial cytoskeleton may contribute to the process. We previously reported that after osmotic stress *in vitro*, the elevated intracellular Ca^{++} concentration in cultured brain microvascular endothelial cells was preserved by KB-R7943. KB-R7943 prevents the removal of elevated intracellular Ca^{++}. The peak level of intracellular Ca^{++} in cultured brain microvascular endothelial cells was not affected by the blocker. In the study presented here, we used KB-R7943 *in vivo* and demonstrated its delaying effect on recovery of the disrupted BBB. The important point of our findings is that this pharmacological manipulation by KB-R7943 did not affect the peak level of PA(s^{-1}). Although the actual level of Ca^{++} and the change in Ca^{++} concentration could not be determined in this *in vivo* study, similar changes in intracellular Ca^{++} *in vitro* and PA(s^{-1}) *in vivo* imply that KB-R7943 exerts its effect via Ca^{++} dynamics *in vivo*.

To date, intracellular signal transduction pathways have been implicated in tight junction formation. Ca^{++} is an important second messenger of intracellular signaling and therefore a rapid increase in intracellular Ca^{++} induced by hyperosmotic conditions may cause drastic changes in cellular functions such as BBB integrity. Two different in vitro models of tight junction assembly in epithelial cells have been used: the Ca^{++} depletion model and the ATP depletion-repletion model. However, epithelial and endothelial cells may assemble and regulate tight junctions differently. Mannitol produced a BBB disruption-closure model which was used in our study as a unique *in vivo* experimental model to study tight junction assembly in brain microvascular endothelial cells.

Ours is the first study in which the rat brain underwent prolonged BBB disruption by the Na^+/Ca^{++} exchange blocker and mannitol. Prolonged BBB disruption may have a deleterious effect on neurons, but our histopathological examination revealed no neuronal damage. The concept of enhanced drug delivery after osmotic disruption is being currently applied to the patients with primary or metastatic brain tumors, resulting in some success and minimal morbidity. With all these clinical implications, pharmacological manipulation of the BBB and its safe limits should be of major interest. Our findings thus represent important experimental information regarding the use of potentially active agents for manipulation of BBB opening for therapeutic purposes.

神戸大学大学院医学系研究科（博士課程）

論文審査の結果の要旨			
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（要旨は１，０００字～２，０００字程度）

脳への水溶性分子の移行は血液脳関門（BBB）の存在によって制限されており脳腫瘍の化学療法や脳への遺伝子導入の妨げとなっている。高浸透圧溶液を用いた脳還流による BBB の可逆的開放は、脳腫瘍治療の臨床にも応用されつつあるが、その機序には未だ不明な点が多い。申請者は高浸透圧刺激による可逆性の BBB 開放の機序を脳還流法を用いて詳細に検討し、Na^+/Ca^{++} exchanger の役割を明らかにした。

方法：

250~300g の全麻下の成熟ラット（52 匹）の外頸動脈から逆行性にカテーテルを挿入し、生理的条件を保ちながら高浸透圧マニトール（1.6M）にて脳を還流することにより、BBB を可逆的に開放した。その後、5 分、15 分、20 分、30 分、60 分の各群で BBB 透過性の経時的変化を測定した。BBB 透過性は ^{14}C-Sucrose を含有した PBS にて脳を３０秒間還流した後、^{14}C-Sucrose の脳内移行を測定する in situ brain perfusion 法を用いた。

Na^+/Ca^{++} exchanger 阻害剤の投与群と非投与群に分けて BBB 透過性の経時的変化を比較検討した。

また、BBB の可逆的開放に Na^+/Ca^{++} exchanger 阻害剤投与を加えることによる脳の組織学的変化の有無を、皮質神経細胞密度を比較することにより検討した。

結果：

(1) Na^+/Ca^{++} exchanger 阻害剤の非投与群において、高浸透圧マニトールによる脳還流の５分後に ^{14}C-Sucrose に対する BBB 透過性は、5.5-5.7 倍に亢進しピークを示した。その後 BBB 透過性は経時的に低下し、６０分後に基礎値に回復した。

(2) Na^+/Ca^{++} exchanger 阻害剤の投与群において、高浸透圧マニトールによる脳還流の５分後の BBB 透過性亢進には変化が無かったが、３０分後には有意に回復が遅延し、６０分後にも 1.7-2.2 倍の亢進を示し基礎値に戻らなかった。こ

の結果は、Na^+/Ca^{++} exchanger 阻害剤は BBB 開放の程度には影響しないが、引き続き生じる BBB の再構築過程を遅延させることを示している。

(3) 高浸透圧マニトール還流側と非還流側の脳における正常神経細胞の密度に有意の差は無く、Na^+/Ca^{++} exchanger 阻害剤投与下の BBB 開放に伴う神経細胞障害は認めなかった。

従来、高浸透圧刺激による BBB の開放は脳微小血管内皮細胞の物理的な収縮によるものと考えられてきた。本研究は BBB の可逆的開放の機序を in situ brain perfusion 法により研究して、この現象には BBB 開放と再構築の異なった２つの相があること、そして BBB 再構築には Na^+/Ca^{++} exchanger の作用が必要であることを初めて明らかにした研究である。従来ほとんど行われなかった高浸透圧刺激による BBB の可逆的開放の機序を解明する上で重要な知見を得たものとして価値ある集積であると認める。よって本研究者は博士（医学）の学位を得る資格があると認める。