Effects of Combination of Vasodilator Drugs and Hypertonic Solutions on Methotrexate Distribution into the Rat Brain

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Abstract—The present experiments were designed to find a method to facilitate methotrexate (MTX) transfer into rat brain tissue. Adult male Wistar-Kyoto strain rats, anesthetized with pentobarbital-Na, received an infusion of the drug solution to be tested into the right internal carotid artery (5 ml/min, 30 sec) 5 min before injection of MTX (7 mg/kg). After 10 min, the MTX levels in the cerebral hemispheres were estimated as a peak height ratio unit of MTX vs. the internal standard by high performance liquid chromatography. MTX was undetectable in either hemisphere after the pretreatment with saline alone or 15% mannitol-saline. The MTX levels in the right hemisphere were about 10 after the pretreatment with 20% mannitol-saline, while MTX was undetectable in the left hemisphere. In contrast, the MTX levels in the right hemisphere were dose-dependently increased to about 25, 130 and 60, 100, respectively, when nitroglycerin (NTG, 2.5 or 7.5 µg/rat) or nicardipine-HCl (NIC, 1.25 or 2.5 µg/rat) was administered together with 20% mannitol-saline. These vasodilator drugs, however, had no effect when tested in combination with saline or 15% mannitol-saline. It is assumed that an increase in cerebrovascular blood flow induced by NTG or NIC enhances the MTX transfer into the brain once the blood-brain barrier is opened by hypertonic solutions.

It is well-known that drugs, especially water-soluble drugs, are hardly distributed into the brain due to the so called blood-brain barrier (BBB). The continuous tight junctions of endothelial cells in the cerebrovascular system have been thought to play the most important role with respect to the barrier (1). It was also reported that even in malignant brain tumors, the BBB may be at least partially intact and restrict drug efficacy (2).

Recently, it was reported that infusion of a hypertonic solution into the internal carotid artery induced a reversible opening of the BBB and increased the brain distribution of systemically-administered drugs in patients with primary and metastatic malignant brain tumors (3) as well as in animals (1, 4–6).

Methotrexate (MTX), the drug used in this study has been mainly used in the treatment of cancers such as leukemia, choriocarcinoma, osteosarcoma and breast cancer. However, because of the low membrane permeability, the efficacy of MTX is said to be weak at the conventional doses given intravenously to patients with cancer. Therefore, based on the assumption that the permeability may increase if the concentration is elevated in extracellular fluids, high doses of MTX have been administered (7). After such treatment, however, normal tissues must be protected from the drug toxicity.

Previously, we have reported that the MTX transfer into the rat brain increased abruptly by intracarotid drug administration after infusion of hypertonic solutions having osmotic pressure above a certain level (8).

Using this method, in the present experiments, we investigated the effects of combination of vasodilator drugs, nitroglycerin (NTG) or nicardipine hydrochloride (NIC), with 20% mannitol-saline on the cerebrovascular permeability to MTX in rats.

Materials and Methods
Male Wistar-Kyoto strain rats, 11–18 weeks old and weighing 290–360 g, were
housed under standard laboratory conditions with water and normal food ad libitum for more than 10 days before use. Under pentobarbital sodium (30 mg/kg, i.p.) anesthesia, a polyethylene catheter was inserted into the trachea, and the animals were allowed to breath room air. A catheter filled with sodium heparin [100 U/ml dissolved in isotonic saline solution (saline)] was retrogradely placed into the right external carotid artery just below the bifurcation of the common carotid artery and used for infusion of test solutions and MTX into the internal carotid artery. This procedure caused no interruption of blood supply to the brain during the experiments. The animals were allowed to rest for 30 min before the experiments.

The drug solutions tested were saline and 15% and 20% mannitol solutions dissolved in saline (15% and 20% mannitol-saline) and these three solutions containing nitroglycerin (NTG, 0.5 or 1.5 µg/ml) or nicardipine hydrochloride (NIC, 0.25 or 0.5 µg/ml).

Each solution, warmed to 37°C, was infused into the right internal carotid artery at a rate of 5 ml/min for 30 sec by means of an infusion pump (type 1830, B. Braun), and a bolus injection of MTX (7 mg/kg, Lederle Japan) in saline was given 5 min afterwards. Ten min later, the right atrium was cut down and saline was infused in the left ventricle until clear perfusate flowed out from the right atrium. Then the brain was removed.

The extraction of MTX from the cerebral hemispheres and its determination were made, respectively, by slight modifications of the method of Buice et al. (9) and that of Deen et al. (10).

The high performance liquid chromatography (HPLC) system consisted of a degasser (Shodex degas model KT-31, Shouwadenkou), a pulseless pump (PM-60, BAS), a syringe loading sample injector (model 17125, Rheodyne), and a variable wavelength detector (UV/VIS monitor, BAS). Ultraviolet absorption was measured at 302 nm. The column (2.6 mm internal diameter X 15 cm length) and pre-column were packed with Zorbax ODS (octadecylsilane, particle size 5 µm, Dupon). The mobile phase consisted of methanol (spectra-analyzed, Wako) and 0.1 M Tris (trishydroxymethylamino-methane, Wako) buffer in a ratio of 1:4 (volume). Before mixing, the buffer was brought to pH 6.5 with 50% phosphoric acid and filtered through a 0.5 µm membrane (Type FH, Millipore) and degassed under a vacuum for 5 min. The flow rate was set at 1.0 ml/min.

Sample preparation and MTX determination in the sample were carried out as follows: Each hemisphere was homogenized in 2 ml of ice cold 10 mM Tris solution with a Potter-Elvehjem homogenizer with a teflon pestle. After centrifugation for 15 min at 3000 rpm, 0.2 ml of the supernatant was transferred to a test tube and mixed with 0.1 ml of 7.4 µM p-aminocetophenone as an internal standard (IS), 0.4 ml of 10 mM Tris solution and 1 ml of distilled water. Then the mixture was passed through the C_{18} Sep-Pak cartridge (Waters) pretreated with 10 ml of methanol and 10 ml of 0.2 M acetate buffer (pH 5.5) by means of a water-jet pump, and followed by 5 ml of acetic acid buffer, 1 ml of 0.1 M sodium hydroxide, and 5 ml of acetate buffer. After the cartridge was dried by further suction, MTX and IS were eluted into a test tube with 2 ml of methanol. The solvent was evaporated to dryness at 60°C with a vacuum centrifugal concentrator (VC-36, Taiyo). The mobile phase (0.1 ml) was added to dissolve the residue, and a 10 µl aliquot was injected into the HPLC. Each retention time was about 5 min for MTX and about 9 min for IS.

The MTX content of each hemisphere was represented in terms of the peak-height ratio unit of MTX vs. IS.

**Results**

Effects of different concentrations of mannitol used for pretreatment are shown in Fig. 1. The MTX levels in both cerebral hemispheres were below the detectable limit in rats pretreated with saline alone and 15% mannitol-saline. On the other hand, the MTX levels were about 10 in the right hemisphere of rats pretreated with 20% mannitol-saline, whereas MTX was undetectable in the left hemisphere. The MTX transfer into the brain was apparent only after the infusion of 20% mannitol-saline.

Effects of NTG or NIC given in combi-
nation with hypertonic mannitol solutions are shown in Fig. 1. The MTX levels in both cerebral hemispheres were below the detectable limit in rats pretreated with saline alone or 15% mannitol-saline containing NTG (7.5 μg/rat) or NIC (2.5 μg/rat). On the other hand, the MTX levels in the right hemisphere were about 100 and 130, respectively, in rats pretreated with 20% mannitol-saline containing NTG (7.5 μg/rat) and NIC (2.5 μg/rat). MTX was also detected in the left hemisphere of these rats, but the levels were all about 10. Both NTG (7.5 μg/rat) and NIC (2.5 μg/rat) enhanced the permeability of cerebral blood vessels to MTX only when these drugs were administered in combination with 20% mannitol-saline.

Effects of different doses of NTG and NIC are shown in Fig. 2. The MTX levels in the right hemisphere were about 25 and 130, respectively, in rats pretreated with 20% mannitol-saline containing NTG (2.5 μg/rat) or NIC (2.5 μg/rat). The effect of 20% mannitol-saline was more markedly augmented when administered in combination with 20% mannitol-saline than 2.5 μg/rat of NTG. The MTX levels in the right hemisphere were increased to about 60 and 100, respectively, in rats pretreated with 20% mannitol-saline containing NTG (2.5 μg/rat) and NIC (2.5 μg/rat) of NIC. NIC also had an effect similar to that of NTG. The permeability of cerebral blood vessels to MTX was increased dose-dependently by NTG (2.5 and 7.5 μg/rat) and NIC (1.25 or 2.5 μg/rat) when these drugs were administered in combination with 20% mannitol-saline.

### Discussion

There were some studies on the osmotic opening of the BBB (1-6). In these studies, 25% mannitol solution was mainly used as a hypertonic solution for pretreatment. However, 25% mannitol solution is unstable, and the precipitates of mannitol are liable to separate even at 37°C. On the other hand, sodium chloride is known as a builder which increases the activities of surface active agents. Therefore, in order to investigate the effects of a combination of polysorbate 80 (Tween 80), a surface active agent, and hypertonic solutions, we primarily used 20% mannitol-saline (8). Twenty percent mannitol-saline is stable at room temperature and a small amount of Tween 80 added to this solution markedly increased the MTX transfer into the brain as compared with this solution alone. From these facts, 20% mannitol-saline was used in the present experiments.

MTX was detected in the right hemisphere of rats pretreated with 20% mannitol-saline, but not with 15% mannitol-saline or saline alone (Fig. 1). From these results, the BBB seems to be opened by pretreatment with 20% mannitol-saline. The BBB-opening effect was markedly augmented by 7.5 μg/rat of NTG or 2.5 μg/rat of NIC given in combi-
nation with 20% mannitol-saline (Fig. 1). Moreover, these combination effects were increased in a dose-dependent manner in the range of 2.5 to 7.5 μg/rat of NTG or 1.25 to 2.5 μg/rat of NIC (Fig. 2). On the other hand, the combination effects were not observed at all when the concentration of mannitol solution was below 15% (Fig. 1).

The cerebral microvessels is morphologically characterized by continuous (non-pored) endothelium, poor-vesicle endothelial cells and continuous tight junctions. The intercellular tight junctions which may play the most important role in BBB is supposed to be opened by solutions with osmotic pressure above a certain level (threshold) (1, 6) such as 20% mannitol-saline and 21% xylitol solution which we reported previously (8).

In conclusion, it was suggested that an increase in the cerebrovascular blood flow induced by vasodilator drugs such as NTG and NIC enhances the MTX transfer into the rat brain when the tight junctions between the cerebrovascular endothelial cells are previously opened by 20% mannitol-saline.

References

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