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Permeability of NS-3, a Thyrotropin-releasing Hormone Analogue, into the Brain After Its Systemic Administration in Rats: A Microdialysis Study

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Abstract

The concentration of NS-3 (montirelin hydrate, CG 3703), a thyrotropin-releasing hormone (TRH) analogue, in the cerebral cortex of urethane-anaesthetized rats was measured after its systemic administration (1 mg kg^{-1}, i.v.), using in-vivo microdialysis coupled with a radioimmunoassay.

The concentration in microdialysates was highest (24 nM) during the first 20 min after injection, and it fell below the detection limit (3·5 nM) 100 min after treatment. The maximal interstitial concentration was estimated to be 0·51 μM.

From these results, it is suggested that NS-3 can readily penetrate into the brain.

Thyrotropin releasing hormone (TRH) is a putative neurotransmitter in the mammalian brain, besides its role as a hypothalamo-pituitary hormone (Oliver et al 1974; Morley 1979; Yarbrough 1983; Horita et al 1986). Immunohistochemical and autoradiographic studies have demonstrated that both TRH-containing neurons and TRH-binding sites are widely but unevenly distributed throughout the brain (Hökfelt et al 1975; Pazos et al 1985). Both systemic and intracerebral injections of TRH produce locomotor hyperactivity, electroencephlographic (EEG) arousal responses and a reversal of pentobarbitone-induced narcosis in rodents (Breeze et al 1975; Horita et al 1976; Beale et al 1977; Kalivas & Horita 1979), thereby suggesting an important role of this peptide in the arousal function. However, such pharmacological actions of TRH are transient due to the rapid metabolism in-vivo by both pyroglutamyl aminopeptidases and prolylendopeptidase (Safran et al 1984; O'Cuinn et al 1990).

NS-3, montirelin hydrate, CG 3703: (3*R*,6*R*)-6-methyl-5-oxo-3-thiomorpholinylcarbonyl-L-histidyl-L-prolinamide tetrahydrate, is a thiomorpholine derivative of the pyroglutamyl moiety of TRH, which shows strong resistance to enzymatic degradation in-vivo (Bauer 1979; Flohé et al 1983). NS-3, like TRH, causes a variety of behavioural arousal responses, such as locomotor hyperactivity and EEG arousal responses (Heal et al 1987; Ukai et al 1988; Higuchi et al 1991; Ogasawara et al 1993), although such actions of this compound were 30–100 times more potent and longer in duration than those produced by TRH. Such potent actions of NS-3 may possibly be due to the favourable permeability of the compound into the brain. However, there is little direct evidence for its penetration through the blood–brain barrier. Therefore, in the present study, we estimated the concentration of NS-3 in the interstitial fluids of the rat cerebral cortex after its systemic injection, using intracerebral microdialysis coupled with a sensitive radioimmunoassay (RIA).

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Materials and Methods

Male Wistar rats (10–11 weeks old, Charles River Japan, Shiga) were housed in a room controlled at 21–25°C, 45–65% relative humidity and maintained in an alternating 12 : 12 light/dark cycle (lights automatically on at 0800 h). Food and water were freely available. Rats were anaesthetized with urethane (1·1 g kg^{-1}, i.p.) and placed on a stereotaxic apparatus (Narishige Scientific Instrument, Tokyo, Japan). The skull was then exposed and a burr hole drilled to insert a dialysis probe into the frontal cortex. The stereotaxic coordinates were 3·7 mm anterior and 3·0 mm lateral to the bregma, and 3·8 mm below the dura mater, according to the atlas of Paxinos & Watson (1982). The dialysis probe used in the present experiment was I-shaped with a 3-mm long cellulose membrane tubing (BDI-I-4-03, Eicom, Kyoto, Japan). The molecular cut-off value of the membrane was 50 000 Da. After implantation of the dialysis probe, Ringer's solution was perfused at a flow rate of 3 μL min^{-1}, using a microinfusion pump (CMA/100, Carnegie Medicine, Stockholm, Sweden). Three hours after the start of perfusion, NS-3 was injected intravenously at a dose of 1 mg kg^{-1}. Microdialysates were collected every 20 min from 1 h before to 2 h after drug injection. In another set of experiments, bolus injection of antipyrine (75 mg kg^{-1}, i.v.) and subsequent continuous infusion of this drug (150 μg kg^{-1} min^{-1} for 80 min) was performed, using another microinfusion pump (EP-60, Eicom), and three consecutive 20-min microdialysate fractions were collected 20–80 min after the start of antipyrine infusion. Blood samples (200 μL) were taken at 30, 50 and 70 min after the start of antipyrine infusion through a polyethylene catheter which was previously implanted into the jugular vein. After centrifugation of blood samples, a portion of plasma was transferred to a 1·5-mL Eppendorf tubing and an equal

volume of 0·4 M perchloric acid was added to precipitate the plasma protein. After centrifugation, the supernatant was diluted with 10 vols distilled water.

Chemicals and drugs used in the present study were NS-3 (montirelin hydrate, CG 3703, Grünenthal GmbH, Aachen, Germany), antipyrine (Nacalai Tesque, Kyoto) and urethane (Sigma Chemical Co., St Louis, MO). Other chemicals were all reagent grade. Drugs were all dissolved in physiological saline.

In-vitro recovery test

In-vitro recovery of NS-3 through the dialysis membrane was tested by immersing the probe into 37°C Ringer's solution containing approximately 1 μM NS-3. One hour after perfusion (3 μL min^{-1}), a 20-min dialysate fraction was collected. For measurement of the in-vitro recovery of antipyrine, the microdialysis probe was placed in 37°C Ringer's solution containing approximately 30 μM antipyrine and normal Ringer's solution was perfused at 3 μL min^{-1}.

Estimation of NS-3 concentration in brain interstitial fluids

The concentration of NS-3 in the interstitial fluid of the rat cerebral cortex after its systemic injection was estimated according to the method of Terasaki et al (1992), using antipyrine as a reference drug. Briefly, the in-vitro permeability rate constant (PA_{vitro}) for NS-3 or antipyrine is shown in equation 1:

$$CL_{vitro} = F \cdot C_d/C_r = F \cdot (1 - e^{-PA_{vitro}/F}) \quad (1)$$

where CL_{vitro} is the in-vitro dialysis clearance, F is the rate of perfusion and C_d and C_r represent the concentrations in microdialysates and reservoir, respectively.

The in-vivo permeability rate constant (PA_{vivo}) is represented by the following equation:

$$CL_{vivo} = F \cdot C_d/C_{isf} = F \cdot (1 - e^{-PA_{vivo}/F}) \quad (2)$$

where CL_{vivo} is the in-vivo dialysis clearance, and C_{isf} represents the concentration in the interstitial fluids.

For antipyrine, which can readily pass through the blood-brain barrier, C_{isf} is presumed to be equal to C_p, the concentration in plasma. Therefore, the PA_{vivo} value for antipyrine can be estimated from C_d and C_p. The effective dialysis coefficient (R_d) is defined as:

$$R_d = PA_{vivo}/PA_{vitro} \quad (3)$$

Based on an assumption that the R_d value for NS-3 is equal to that for antipyrine, the interstitial concentration of NS-3 in the cerebral cortex can be deduced as follows:

$$C_{isf.NS\text{-}3} = C_d/(1 - e^{-R_d} \cdot PA_{vitro,NS\text{-}3}/F) \quad (4)$$

Determination of NS-3

NS-3 in microdialysates was determined by RIA. The detailed procedure for NS-3 assay is shown in Fig. 1.

Determination of antipyrine

The concentrations of antipyrine in microdialysates and plasma were determined by high performance liquid chromatography (HPLC) with UV spectrophotometric detection, according to the method of Deguchi et al (1991) with slight modifications. The HPLC system was composed of a pump (LC-6A, Shimadzu, Kyoto), a degasser (DG-100, Eicom), a sample injector (Model 7125, Rheodyne), a guard column (5 × 4·6 mm, inside diameter) packed with Nucleosil 5C18 (Chemco, Osaka, Japan), a reversed-phase separation column (Capcell Pak C18, 150 × 4·6 mm, inside diameter, Shiseido, Tokyo), a chromatocorder (C-R3A, Shimadzu) and a UV-spectrophotometer (SPD-6A, Shimadzu). The mobile phase was 0·1 M potassium phosphate buffer (pH 7·2) containing 22% acetonitrile. The flow rate was 0·8 mL min^{-1}, and the detection wavelength was 254 nm.

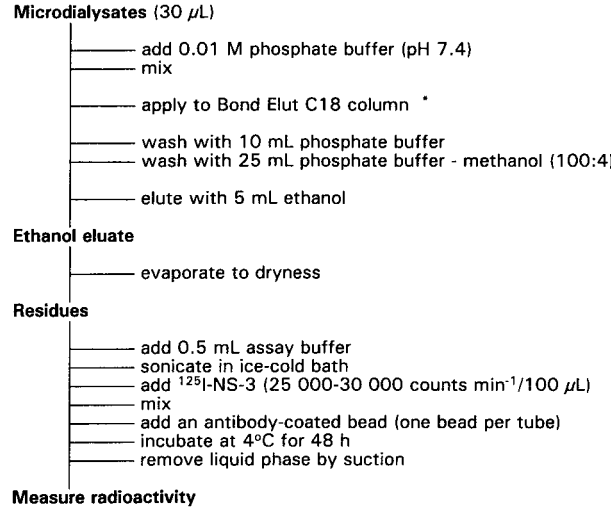

FIG. 1. Assay procedure for NS-3 by RIA. [a]0·01 M phosphate buffer (pH 7·4). *The column was washed with 5 mL ethanol, followed by 5 mL phosphate buffer, 20 mL ethanol and then 10 mL phosphate buffer prior to application of samples.

Results

The present RIA method enabled determination of concentrations of NS-3 as low as 0·05 ng in 30 μL (3·5 nM). As

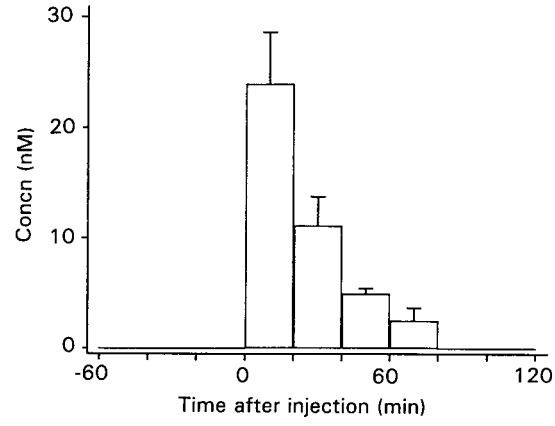

FIG. 2. Concentrations of NS-3 in microdialysate fractions of the rat cerebral cortex after systemic injection of NS-3. Rats were anaesthetized with intraperitoneal urethane (1·1 g kg^{-1}) and placed on a stereotaxic apparatus. A microdialysis probe was inserted into the cerebral cortex. Two hours after start of perfusion, microdialysates were collected every 20 min. NS-3 (1 mg kg^{-1}) was injected, and determined in the microdialysates by RIA. Each column represents the mean ± s.e.m. of five experiments.

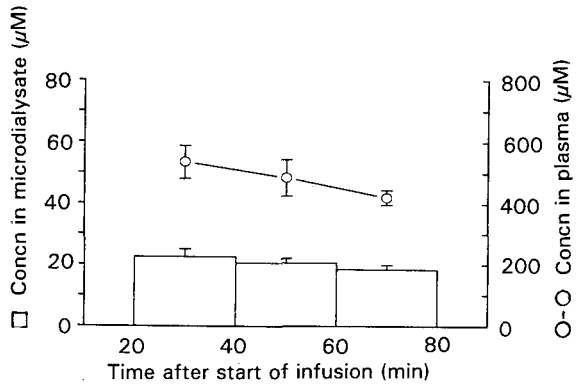

FIG. 3. Concentrations of antipyrine in plasma and microdialysate fractions of the rat cerebral cortex during systemic injection of antipyrine. Antipyrine (75 mg kg^{-1}) was administered intravenously as a bolus followed by continuous injection via the tail vein at 150 μg kg^{-1} min^{-1} for 80 min. Blood samples were taken from the jugular vein 30, 50 and 70 min after start of antipyrine infusion. Microdialysates were obtained every 20 min after start of antipyrine infusion. Each point represents the mean ± s.e.m. of four experiments.

shown in Fig. 2, NS-3 was detected in microdialysates of the rat cerebral cortex after its intravenous injection of 1 mg kg^{-1}, although there were no detectable NS-3-like immunoreactivities in the microdialysates obtained before NS-3 treatment. The concentration of NS-3 was maximal (24 nM) during the first 20 min after injection, and it was below the detection limit 100 min after treatment. The in-vitro recovery of NS-3 through the dialysis membrane was 8·3%, and the PA_{vitro} value, which was obtained from equation 1 as described in Materials and Methods, was 0·26 ± 0·02 μL min^{-1} (mean ± s.e., n = 5).

To estimate the interstitial concentration of NS-3, we measured the in-vitro and in-vivo dialysis probe recovery using antipyrine as a reference drug. Fig. 3 shows the concentrations of antipyrine both in the plasma and in the cortical microdialysates during an intravenous bolus antipyrine injection (75 mg kg^{-1}) followed by continuous infusion at 150 μg kg^{-1} min^{-1} for 80 min. The PA_{vivo} value obtained from equation 5 was 0·13 ± 0·01 μL min^{-1} (n = 4). From the in-vitro recovery test, the PA_{vitro} value for antipyrine was 0·25 ± 0·04 μL min^{-1} (n = 4). Therefore, the R_d value was 0·55 ± 0·05.

Based on the assumption that the R_d values of NS-3 and antipyrine are equal, the brain interstitial concentrations of NS-3 were estimated from equation 4. The maximal concentration of NS-3 in the interstitial fluid (during the first 20-min fraction after injection) was estimated to be 0·51 ± 0·10 μM.

Discussion

In the present study, we used intracerebral microdialysis to investigate the permeability of NS-3 into the rat brain. This technique, which was originally developed by Bito et al (1966) and later modified by Ungerstedt et al (1982) to measure the extracellular concentration of various neurotransmitters in brains of living animals (Ungerstedt & Hallström 1987), has now been applied to a variety of studies, including pharmacokinetic experiments (Benveniste 1989; Benveniste et al 1989; Terasaki et al 1991). In contrast to other perfusion methods, such as push-pull perfusion, this technique does not cause serious tissue damage, since the perfusion fluid does not come into direct contact with brain tissues. Benveniste (1989) has demonstrated in the rat striatum that the function of the blood-brain barrier almost completely recovered 30 min after implantation of the dialysis probe, using α-amino-*iso*-butyric acid, a non-metabolizable amino acid which hardly penetrates into the brain. Similar results were obtained by Terasaki et al (1992), who reported, using [^{14}C]sucrose, that the blood–brain barrier is almost intact between 1 and 48 h after implantation of the dialysis probe into the rat hippocampus. In the present study, drugs were injected 3 h after insertion of the dialysis probe.

In the present NS-3 assay by RIA, the anti-NS-3 antibody showed substantial cross-reactivity with the de-amide metabolite of NS-3, CNK-6004, although the antibody hardly recognized TRH. Therefore, before NS-3 assay the microdialysates were applied to a column packed with reversed-phase resin to remove CNK-6004. In addition, there was no detectable NS-3-like immunoreactivity in microdialysates before injection of NS-3. Taken together, it is suggested that the NS-3-like immunoreactivity observed in the present study was mostly derived from NS-3 itself. After intravenous injection of NS-3 at 1 mg kg^{-1}, the immunoreactivity was detected in microdialysates obtained from the rat cerebral cortex. The maximal concentration was observed in the first 20-min fraction (24 nM), and the level was below the detection limit (3·5 nM) 100 min after treatment. Therefore, the present findings clearly demonstrated the penetration of NS-3 into the brain after its systemic injection.

In the microdialysis experiment, the interstitial concentration could be estimated by the recovery method and the reference method (Benveniste 1989; Terasaki et al 1992). The former is simple, involving estimation only from its in-vitro recovery. However, the interstitial concentration calculated by the former method may be underestimated since the in-vivo probe recovery is always lower than the value obtained in-vitro (Benveniste et al 1989; Terasaki et al 1992). Therefore, we estimated the interstitial concentration of NS-3 in the rat cerebral cortex by the reference method reported by Terasaki et al (1992), using antipyrine as a reference drug. The brain interstitial concentration of NS-3 was assumed to be 0·51 μM during the first 20 min after its systemic injection at 1 mg kg^{-1}. However, several presumptions were made for evaluation of extracellular NS-3 concentration. First, the PA_{vitro} value for antipyrine was obtained based on the assumption that the interstitial concentration is equal to that in plasma. Second, the ratio of PA_{vivo} to PA_{vitro} (R_d) for antipyrine may not be different from that for NS-3.

We have recently found that NS-3 produced an enhancement of acetylcholine release from the rat cerebral cortex, as studied by intracerebral microdialysis (Itoh et al 1994). The increase in the cortical acetylcholine release after intravenous injection of 0·1 mg kg^{-1} of NS-3 was to almost the same extent as that observed after its application at a concentration of 1 μM via the dialysis probe into the nucleus basalis magnocellularis, where cell bodies of acetylcholine containing neurons projecting to the cerebral cortex exist

(Mesulam et al 1979). The interstitial concentration of NS-3 after its systemic injection at 0·1 mg kg^{-1} or its perfusion into the nucleus basalis magnocellularis at 1 μM would be approximately 0·051 or 0·046 μM, respectively, which were calculated from the present experimental data, including the extracellular concentration (0·51 μM) after its injection at 1 mg kg^{-1}, the in-vitro recovery of NS-3 (8·3%) and R_d value (0·55). Therefore, the present findings may reasonably explain such pharmacological actions of NS-3.

In conclusion, we have measured the extracellular concentration of NS-3 after its systemic injection, using intracerebral microdialysis coupled with RIA. The maximal microdialysate concentration was 24 nM, which was observed during the first 20 min after treatment. According to the reference method using antipyrine, the interstitial concentration was assumed to be approximately 0·51 μM. Thus, we concluded that NS-3 readily passes through the blood–brain barrier.

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