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In Vivo Measurement of Extracellular Monoamines and Their Metabolites in the Rat Posterior Hypothalamus Using Microdialysis Technique

Ki Wug Sung, Seong Yun Kim, Young Jin Cho, Kweon Haeng Lee and Sang Bok Lee*

Department of Pharmacology, Catholic University Medical College, Seoul, Korea

ABSTRACT

Catecholamines, serotonin and their metabolites were measured in the posterior hypothalamus of urethane-anesthetized normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) using brain microdialysis which is a recently developed experimental method to measure the release of neurotransmitters and their metabolites at the localized brain area in vivo. Microdialysis probe was implanted stereotaxically to the rat posterior hypothalamus and perfused by Ringer's solution. Monoamines and their metabolites were quantified by reverse phase high performance liquid chromatography with electrochemical detection. *In vitro* recovery test of microdialysis showed that there exist inverse relationship between the perfustion flow rate and the relative recovery of neurochemical compounds. The estimated extracellular concentration of dopamine was about 32 nM, of norepinephrine 50 nM, of epinephrine 50 nM, of serotonin 73 nM, of 3, 4-dihydroxyphenylacetic acid (DOPAC) 281 nM, of homovanillic acid (HVA) 181 nM, and of 5-hydroxyindoleacetic acid (5HIAA) 3767 nM in the hypothalamic perfusate of the normotensive rat. There was no difference in the basal lelvel of monoamines between the SHR and the WKY. In contrast, the level of DOPAC, HVA and 5HIAA in SHR was higher than that in the WKY, This study demonstrated that the microdialysis technique should be an applicable tool for *in vivo* measurement of central neurochemical substances.

Key Words: Brain *in vivo* microdialysis, Posterior hypothalamus, Catecholamines, Serotonin, Spontaneously hypertensive rats

INTRODUCTION

Brain monoaminergic systems may play a role in the central regulation of cardiovascular system (Chalmers, 1975; Axelrod, 1976; Kuhn *et al.*, 1980). The majority of these brain monoaminergic cell bodies are located in the lower brain stem where they project to all parts of the central nervous system (Hökfelt *et al.*, 1974; Dahlström and Fuxe, 1964). The action sites of central monoamines in the cardiovascuar regulation are probably in hypothalamus and brain stem areas (Axelrod, 1976; Chalmers, 1975; Loewy and Neil, 1981).

There is considerable evidence that in the spontaneously hypertensive rats (SHR), an useful experimental model for human essential hypertension (Trippodo and Frohlich, 1981), imbalance in a number of neurotransmitter systems in the central nerovus system may influence sympathetic outflow and contribute to the development and maintenance of hypertension (Okamoto *et al.*, 1967; Louis *et al.*, 1987). Many investigators have reported that there is a significant difference between the concentrations of monoaminergic neurotransmitters in the various brain areas of the SHR and

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*To whom reprint requests should be addressed.

normotensive Wistar-Kyoto rats (WKY) (Saavedra *et al.*, 1978; Wijnen *et al.*, 1980; Howe *et al.*, 1981; Koulu *et al.*, 1986; Kubo *et al.*, 1990). But, the majority of current knowledge in this field have stemed from *in vitro* experiments using postmortem tissues.

Recently, brain *in vivo* microdialysis is introduced as a powerful new technique for monitoring of events occurring in the extracellular fluid including neurotransmission and the relavant metabolism (Ungerstedt, 1984).

Therefore, the present study was undertaken to measure the extracellular concentration of endogenous monoamines and their metablites in the posterior hypothalamus *in vivo* of SHR as well as WKY as control. This was done using *in vivo* microdialysis technique combined with high preformance liquid chromatography and electrochemical detection (HPLC-ECD).

MATERIALS AND METHODS

General procedure

Experiments were performed on male SHR (12~17 weeks) and age-matched WKY controls. These animals were obtained from the Charles River Japan Inc. (Japan) and they were maintained by brother-sister breeding in our laboratory. The animal was anesthetized with urethane (1.2 g/kg, i. p.) and maintenance dose of α-chloralose (20 mg/kg, i. v.) being given when required. After the trachea was cannulated, polyethylene catheter was placed in the left femoral vein for the i. v. injections. The animal was paralyzed with d-tubocurarine (0.5 mg/kg, i. m.) and artificially ventilated with room air enriched with O_2 using a rodent ventilator (Harvard, Model 680, USA). Rectal temperature was maintained between 37±0.5°C with a thermostatically controlled heating plate.

Brain microdialysis

The rat was placed in a stereotaxic instrument (David Kopf Instruments, USA) in a prone position with the upper incisor bar 3.3 mm below the interaural line and the skull exposed. A small hole was drilled for the stereotaxic implantation of a microdialysis probe (Carnegie Medicin, CMA/10, Sweden) into the posterior hypothamalus (coordinates: rostral -4.3 mm, lateral 0.5 mm, ventral 8.0 mm, relative to bregma and brain surface). The coordinates were chosen according to the stereotaxic atlas of Paxinos and Watson (1986). The probe was perfused during implantation and throughout the subsequent experimental period with Ringer's solution (147 mM NaCl, 2.3 mM $CaCl_2$, 4 mM KCl, pH 7.4, flow rate 0.75 μl/min) via polyethylene tubing connected to a 1 ml syringe mounted on a microinfusion pump (Carnegie Medicin, CMA/100, Sweden). The dialysate was then collected in 20-min fraction in 250 μl Eppendorf tube containing 10 μl of 0.4 M perchloric acid using a fraction collector (Carnegie Medicin, CMA/140, Sweden) (Fig. 1). When the level of monoamines and metabolites in the dialysate was stable after probe implantation, three 20-min fractions of dialysate were collected to measure the basal level of monoamines and metabolites. All dialysates were analysed for monoamines and metabolites on the day of the experiment without any further preparation or freezing.

In vitro recovery test

Estimates of the recovery of the monoamines and metabolites through microdialysis probe were conducted *in vitro*. In this experiment, the dialysis probe was placed in a 1.5 ml Eppendorf tube containing the relavant monoamines and metabolites in dissolved Ringer's solution (10^{-7} M of each substance) and then perfused Ringer's solution at flow rate 0.75, 1.0, 1.5, 2.0 μl/min. 20-min dialysis samples were collected and assayed by HPLC-ECD. In the same perfusion and chromatographic conditions stated below, the amount of each compound in the dialysate was compared with the amount in the Ringer's solution and expressed as percentage of recovery. At a perfusion rate 0.75 μl/min, the extracellular concentration of substances was calculated as

$$C_{ext} = C_{dial} / \text{recovery rate } \textit{in vitro}$$

;C_{ext}, estimated extracellular concentration of substances in the posterior hypothalamus; C_{dial}, concentration of substances in the dialysate taken from the posterior hypothalamus.

HPLC-ECD analysis of monoamines and metaboiites in dialysate

Twenty-minute dialysis samples were analyzed without any purification procedures. The mono-

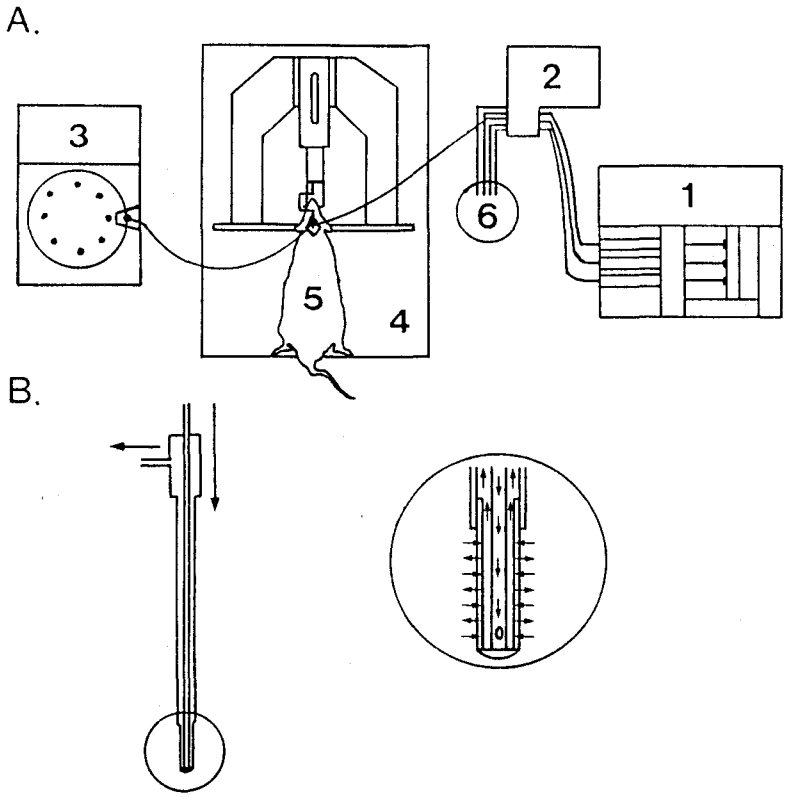

Fig. 1. Schematic diagram of microdialysis system(A) and microdialysis probe(B). 1, Microinfusion pump; 2, Syringe selector; 3, Microfraction collector; 4, Stereotaxic instrument; 5, Animal implanted with a microdialysis probe; 6, Waste

amines and their metabolites were separated by ion pair reverse phase chromatography (μBondapak C_{18}, 30 cm×3.9 mm, Waters, USA), which was maintained at 30°C with column heater (Waters, USA). The mobile phase consisted of 0.05 M citric acid, 0.05 M disodium phosphate (pH 3.1), 3.2 mM 1-octanesulfonic acid (sodium salt), 0.3 mM EDTA and 14% methanol and was pumped at a flow rate of 1.0 ml/min using a solvent delivery system (Waters, M510, USA). Samples (20 μl) were injected with a Waters U6K injector and the compounds were detected coulometrically (ESA, Model 5100A, USA) with an analytical cell (ESA, Model 5010, USA). Potential for the first and second cell was set at+0.01 V and+0.32 V, respectively, A guard cell (EAS, Model 5020, USA; potential+0.45 V) was placed before injector. Chromatograms (Fig. 2) were displayed and analysed on a chromatographic integrator (Spectra-physics, Chromjet, USA). The concentrations of monoamines and metabolites detected in the dialysate samples were determined by comparison with standard solution of norepinephrine (NE), epinephrine (EPI), dopamine (DA), serotonin (5HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5 HIAA) (0.4, 0.4, 0.4, 0.4, 0.8, 0.8, 8.0 pmol, respectively) injected into the column immediately before and after each experiment.

Histology

At the end of the experiment, the microdialysis probe was removed and the brain was perfused intracardially with saline followed by 10% phosphate-buffered formalin. Coronal sections (50 μm) of the brain were made on a cryostat (Reichert-Jung, FRG) and stained with cresyl violet. Photo-

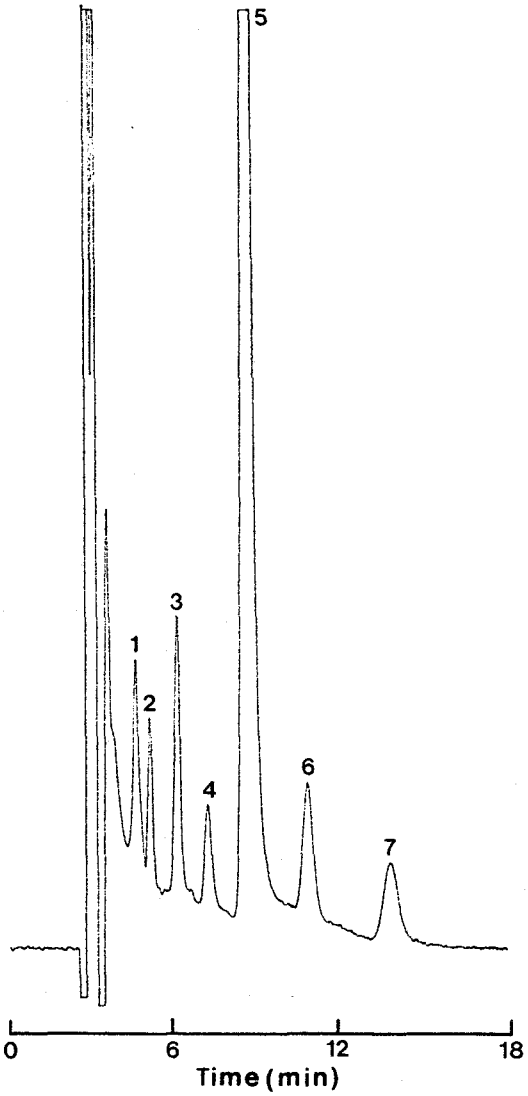

Fig. 2. Typical chromatogram of monoamines and metabolites present in a standard solution. 1, norepinephrine 0.4 pmol; 2, epinephrine 0.4 pmol; 3, DOPAC 0.8 pmol; 4, dopamine 0.4 pmol; 5, 5HIAA: 8 pmol; 6, HVA 0.8 pmol; 7, serotonin 0.4 pmol

graphs of the sections on slides were made and the implantation site of dialysis probe was verified histologically, using the stereotaxic atlas of Paxinos and Watson (1986).

Data presentation and statistical analysis

Data presented are expressed as mean±S. E.. Difference between the values form the SHR and the WKY were compared by Student's t-test for unpaired data. Statistical significance was accepted for $p<0.05$.

Drugs and reagents

Monoamine standards (NE hydrochloride, EPI bitartrate, DA hydrochloride. 5HT creatine sulfate, DOPAC, HVA, 5HIAA), reagent grade of sodium chloride, potassium chloride, perchloric acid, disodium EDTA, calcium chloride dihydrate and 1-octanesulfonic acid sodium salt were purchased from Sigma Chemical Company (St. Louis, USA); citric acid monohydrate and HPLC grade of methanol from Fisher Scientific (Fair Lawn, USA); sodium phosphate 12 H_2O from Wako Chemicals (Osaka, Japan).

RESULTS

***In vitro* recovery test**

To estimate the relative recovery rate, *in vitro* recovery tests were performed in various flow rates (0.75, 1.0, 1.5, 2.0 μl/min) at room temperature. Table 1 shows the relationship between perfusion flow rate and relative recovery of monoamines and metabolites across dialysis membrane. When the flow rate was increased, the relative recovery rate for each substance was decreased.

Esimated extracellular levels of monoamines and metabolites

The level of monoamines and their metabolites in the dialysate was routinely stable 2hr after probe implantation. Table 2 shows the level of monoamines and metabolites in the dialysates obtained from the posterior hypothalamus, relative recovery rate *in vitro* at flow rate 0.75 μl/min and estimated extracellular level of monoamines metabolites in the posterior hypothalamus.

Difference in levels of monoamines and metabolites between WKY and SHR

Fig. 3 and Fig. 4 summarize the result of meas-

Table 1. Effect of perfusion flow rate on *in vitro* recovery of amines and metabolites across the dialysis membrane

Compound	Recovery (%) at flow rate (μl/ml) of			
	0.75	1.0	1.5	2.0
NE	22.5±1.3	18.8±1.2	14.9±0.9	14.4±1.0
EPI	21.4±2.3	17.5±2.3	13.9±1.8	11.1±1.3
DA	20.9±1.7	18.3±1.3	15.2±2.2	9.6±0.4
5HA	12.7±2.8	7.4±1.0	6.7±0.7	6.5±0.4
DOPAC	22.3±1.4	19.8±1.8	15.8±1.8	14.4±1.8
HVA	21.0±1.0	17.4±1.3	12.2±0.9	9.8±0.7
5HIAA	10.3±3.4	5.9±1.4	5.9±1.3	6.0±1.5

The microdialysis probe was placed in a Eppendorf tube containing a mixture of norepinephrine(NE), epinephrine (EPI), dopamine(DA) serotonin(5HT), DOPAC, HVA and 5HIAA, 10^{-7} M each. Values are expressed as mean±S. E. (N=8).

Table 2. Estimated extracellular concentrations of monoamines and metabolites in the posterior hypothalamus of the normotensive rat.

Compound	C_{dial} (pmol/15 μl)	Recovery (%)	C_{ext} (nM)
NE	0.17	22.5	50
EPI	0.16	21.4	50
DA	0.10	20.9	32
5HT	0.14	12.7	73
DOPAC	0.94	22.3	281
HVA	0.57	21.0	181
5HIAA	5.82	10.3	3767

Values are expressed as mean(N=8).
C_{dial}, concentration in the dialysate; C_{ext}, estimated extracellular concentration

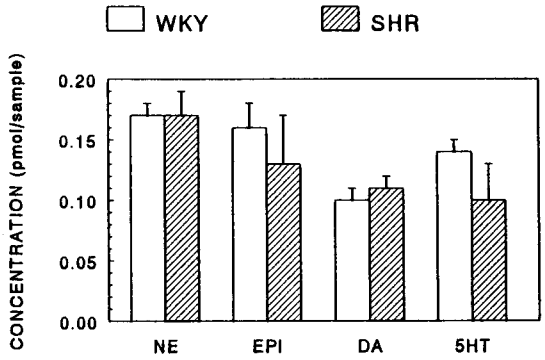

Fig. 3. Basal release of norepinephrine(NE), epinephrine(EPI), dopamine(DA) and serotonin(5HT) into dialysate(15 μl/20 min) from the posterior hypothalamus of the WKY and the SHR, Each group consists of 8 rats. The values are mean± S. E..

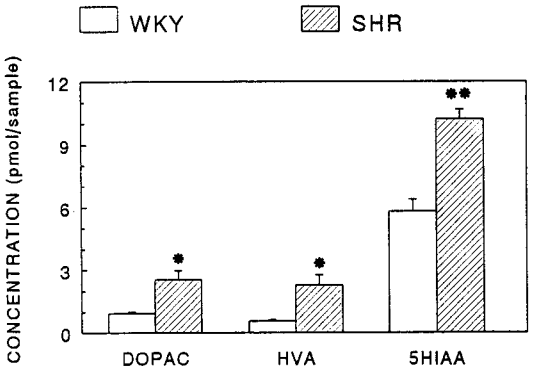

Fig. 4. Basal release of DOPAC, HVA and 5HIAA into the dialysate(15 μl/20 min) from the posterior hypothalamus of the WKY and the SHR. Each group consists of 8 rats. The values are mean± S. E..
*p<0.01, **p<0.001 as compared with the WKY

urements of monoaminergic neurotransmitters (NE, EPI, DA and 5HT) and their metabolites (DOPAC, HVA and 5HIAA) content of dialysate sample, respectively. There was no significant difference in NE, EPI, DA, 5HT levels at the posterior hy-pothalamus between the WKY and the SHR (Fig. 3). The DOPAC, HVA and 5HIAA levels of the posterior hypothalamus were significantly greater in the SHR as compared to the WKY (Fig. 4).

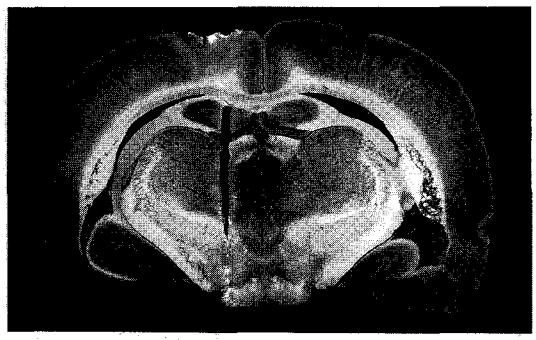

Fig. 5. Photograph of a coronal section of the brain at the level of posterior hypothalamic area. The track of microdialysis probe is in the dorsoventral direction. The photograph is of a 50 μm section stained with cresyl violet.

Histological verification of an implantation site of dialysis probe

Figure 5 shows the brain section at the level of posterior hypothalamic area in one of the experiments. The area where the dialysis probe was implanted corresponded to the posterior hypothalamus as compared with the rat brain atlas of Paxinos and Watson (1986).

DISCUSSION

The present study demonstrates that we are able to measure the release of endogenous DA, EPI, NE and 5HT as well as extracellular levels of their metabolites DOPAC, HVA and 5HIAA in the rat posterior hypothalmus *in vivo* using brain microdialysis combined to HPLC-ECD. Furthermore, the present results provide the first *in vivo* evidence that the extracellular levels of DOPAC, HVA and 5HIAA in the posterior hypothalamus of the SHR were higher than those of the WKY.

A new technique, *in vivo* microdialysis, is becoming widely used to identify extracellular neurochemical events in the regional brain areas (Ungerstedt, 1984). The basic principles of brain microdialysis is the positioning of a small-diameter dialysis membrane that allows free diffusion of water and solutes between the solution lacking the substances concerned. This solution is constantly renewed and sampled for further analysis (Benveniste, 1989). The major advantages of brain dialysis over other techniques for *in vivo* monitoring of neurochemical chages on the brain (cup technique, push-pull technique) stem from the fact that it is a closed system in which there is no direct contact between superfusion fluid and tissue; thus local tissue damage is reduced and relatively clean smaples are collected for direct chemical analysis (Di Chiara, 1990). In the present study, the microdialysis probe with a 2.0 mm length of dialysis tubing was initially tested *in vitro* to determine the effect of flow rate on the recovery of monoamines and their metabolites. The term 'recovery' is defined as the ratio between the concentration of a particular substance in the outflow solution and the concentration of the same substance in the solution outside the probe (Ungerstedt *et al.*, 1982). The present results are in agreement with the findings of Nakahara *et al.*, (1989), who reported that the relative recovery of neurochemical compound declined with increased flow rate.

Different results (Fairbrother *et al.*, 1990; Matos *et al.*, 1990; Santiago and Westerink, 1991) have been reported regarding *in vitro* recovery of neurochemical compounds by the dialysis probes, probably depending on several factors including perfusion flow rate, temperature, diffusion coefficients of various substances measured and dialysis membrane area (Benveniste, 1989). Our result showed that the *in vitro* recovery of catecholamines and their metabolites is higher than that of indolamine and its metabolite. This result is consistent with a previous report demonstrating that the *in vitro* recovery of 5HT and 5HIAA among catecholamines, serotonin and their metabolites had the lowest (Hernandez *et al.*, 1986).

We found that the basal level of monoaminergic substances was stable 2 hours after the implantation of dialysis probe, being consistent with numerous studies (Sharp *et al.*, 1989; Moghaddam *et al.*, 1990; Anderson and DiMicco, 1990). Also, our data that estimated extacellular levels of amines and metabolites in the posterior hypothalamus are tens nanomolar range for NE, EPI and 5HT, a few hundred nanomolar range for DOPAC and HVA, and a few micromolar range for 5HIAA, are in general agreement with the findings of Routledge and Marsden (1987). In addition, consistent with our results, numerous studies showed that extra-

cellular levels of metabolites are much greater than those of their substrates neurotransmitters (Sharp *et al.*, 1986; Carrozza *et al.*, 1991; Gardier and Wurtman, 1991). It is important that for proper functioning of neurotransmitter-receptor interactions, neurotransmitters are effectively removed from the synaptic extracellular fluid. Highly effective uptake mechanisms and the presence of metabolizing enzymes are thought to be responsible for a rapid clearance of the transmitter from the extracellular fluid (Horn *et al.*, 1971). A high metabolite/neurotransmitter ratio is therefore a characteristic of the extracellular level of monoaminergic neurotransmitters.

Several lines of evidence suggested that there exist catecholaminergic and serotonergic cell bodies or nerve terminals in the posterior hypothalamus (Dahlström and Fuxe, 1964; Swanson and Hartman, 1975; Steinbusch, 1981; Swanson, 1982; Vertes *et al.*, 1986). An interesting result from this study is that the extracellular levels of DOPAC, HVA and 5HIAA in the posterior hypothalamus of the SHR were higher than those of the WKY, while the extracellular levels of DA, NE, EPI and 5HT were not the case. There is biochemical evidence that the urinary level, reflecting blood level, of HVA does not differ between the SHR and the WKY (Louis *et al.*, 1970; Racz *et al.*, 1986). Thus, it is unlikely that monoamines and metabolites measured in this study were originated from blood. Related findings were reported in a previous study showing that in the SHR, altered neurotransmission in the posterior hypothalamus may be a trigger mechanism for the development of hypertension (Winternitz *in vivo* 1984). But, the significance of our result remains to be further investigated.

In concluion, this study demonstrated that the microdialysis technique should be an applicable tool for *in vivo* measurement of the central neurochemical substances.

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= 국문초록 =

미세투석법을 이용하여 흰쥐 후 사상하부에서 세포외액의 모노아민과 대사체들의 생체내 측정

가톨릭대학교 의학부 약리학교실

성기욱 · 김성윤 · 조영진 · 이권행 · 이상복

최근에 개발된 생체내 미세투석법을 이용하여 정상혈압 흰쥐(WKY)와 자연발생성 고혈압 흰쥐(SHR)의 후 시상하부에서 세포외액의 모노아민과 그 대사체들을 측정하였다. 뇌정위 고정장치에 의해서 미세투석관을 후 시상하부에 위치시킨후 링거액으로 관류하였다. 모노아민과 그 대사체들은 고속액체 크로마토그라피와 전기화학 검출기를 이용하여 정량하였다. 미세투석관의 시험관내 회수율 검사 결과, 관류액의 유속과 신경화학물질의 상대적 회수율 사이에는 역비례 관계가 있음이 확인되었다. 정상 혈압 흰쥐에서 후 사상하부의 관류액으로 부터 측정한 각종 신경화학물질의 세포외액 농도는 도파민 32 nM, 노르에피네프린 50 nM, 에피네프린 50 nM, 세로토닌 73 nM, 3.4-dihydroxyphenylacetic acid(DOPAC) 281 nM, homovanillic acid(HVA) 181 nM, 5-hydroxyindoleacetic acid(5HIAA) 3767 nM이었다. 후 시상하부에서 측정된 신경전달물질의 기준치는 WHY와 SHR사이에 차이가 없었으나, DOPAC, HVA, 5HIAA의 기준치는 WKY에 비해서 SHR에서 유의하게 높게 나타났다. 본 연구는 중추 신경화학물질들의 생체내 측정에 미세투석법을 이용할 수 있음을 보여주었다.