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Determination of myocardial norepinephrine in freely moving rats using in vivo microdialysis sampling and liquid chromatography with dual-electrode amperometric detection

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Abstract

Myocardial norepinephrine (NE) is considered a meaningful parameter for estimation of cardiac function. Long lasting changes in myocardial NE appear to be not only a consequence of pathologic processes in the myocardium, but may be a factor responsible for some diseases (e.g. increased propensity for arrhythmias or negative effect on left ventricular contractility in congestive heart failure). In this respect monitoring of myocardial NE is of great importance. A microdialysis sampling technique coupled with liquid chromatography with electrochemical detection (LCEC) was developed to measure the in vivo NE concentration in the myocardial interstitium of conscious, freely moving rats. LCEC using a dual-electrode amperometric detection in the series configuration provided detection limits for NE of 10 pg/ml in 20 μl microdialysis samples. Microdialysis probes of the linear design were implanted in the myocardial tissue in the periphery of the left descending coronary artery. The basal steady-state concentration of NE in myocardial dialysate of awake, freely moving rats was found to be 0.17 ± 0.026 ng/ml. Delivery through the microdialysis probe of the NE reuptake inhibitor desipramine (DMI) at a concentration of 0.1 mM increased NE release to $153 \pm 13\%$ of control. If the concentration of DMI in the perfusate was increased to 1.0 mM, NE release increased to only $166 \pm 21\%$ of control.

Keywords: Myocardium; Norepinephrine; Liquid chromatography with electrochemical detection; Microdialysis; Myocardial ischemia

1. Introduction

It has been established that changes in the level of myocardial norepinephrine (NE) are closely related to changes in cardiac functioning. A marked increase in myocardial NE has been observed after acute myocardial ischemia [1–3]. A decrease in myocardial NE has been shown to accompany long-term ischemia, cardiomyopathy, and various forms of heart failure [4–6]. The decrease of myocardial NE appears to be not only a consequence of pathologic processes in the my-

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ocardium, but may also be a factor responsible for the increased propensity for arrhythmias [5] and for the negative effect on left ventricular contractility in congestive heart failure [7]. These reports show the importance of extended study of changes in myocardial NE under the influences of different physiological factors as well as pharmacological agents.

The use of microdialysis sampling to monitor NE in the myocardial interstitium has been previously reported [2,6,8–13]. Most of these studies were performed in comparatively large experimental animals: cats, rabbits or pigs. This allowed the use of long microdialysis probes for increased NE recovery in the myocardial dialysate. These experiments were also performed under general anesthesia. It has been shown recently that anesthesia can influence myocardial sympathetic neuronal network, considerably changing its reaction to a repeated ischemic stressor [14]. Cremers et al. [13] have reported the use of microdialysis sampling from the myocardium of conscious rats.

In this report we describe the development of a microdialysis sampling technique to continuously monitor myocardial norepinephrine in conscious, freely moving rats. A linear microdialysis probe was employed to minimize tissue damage while providing a secure implantation. Norepinephrine was detected in dialysis samples by dual-electrode amperometric detection following HPLC separation. The utility of this technique was demonstrated by studying NE release during delivery of the NE reuptake inhibitor desipramine (DMI).

2. Experimental

2.1. Chemicals

Sodium octylsulphonate, norepinephrine, and perchloric acid (99%) were obtained from Sigma (St. Louis, MO). EDTA and HPLC grade methanol were purchased from Fisher Scientific (St. Louis, MO). All chemicals were reagent grade or better and used as received.

2.2. Microdialysis probe design

Linear microdialysis probes were used in these studies. The active dialysis window of the probe consisted of polyacrylonitrile (PAN) hollow fiber (240 μm I.D. × 340 μm o.d., MWCO 29000, CGH Medical, Inc., Lakewood CO) with a length of 5–6 mm. Flexible polyimide capillary (160–200 μm i.d., 200–240 μm o.d. (HV Technologies, Inc. Trenton, GA) was used as the inlet and outlet tubing. These capillaries were inserted into the dialysis membrane and cemented by UVE-4106 epoxy adhesive (Star Technology, Inc., Waterloo, IN). An 80-μm o.d. nylon fiber was inserted throughout the length of the microdialysis fiber to provide mechanical strength. The outlet tubing was attached to a 25-gauge hypodermic needle as a guide during probe implantation. A Tygon ring was mounted on the distal end of the microdialysis membrane using UV epoxy to restrict its movement in the myocardium. Before implantation the probe was flushed with methanol for 20 min at a flow rate of 10 μl/min while the probe was immersed in distilled water. The probe was then flushed with distilled water for 2 min.

2.3. Microdialysis probe characterization

Microdialysis probes were calibrated in vitro by placing them in a standard solution of 1 ng/ml NE in Ringer's solution maintained at 37°C. Ringer's solution consisted of 145 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ and was treated with Chellex-100 (Bio-Rad, Richmand, CA) to remove iron prior to use. The probe was perfused at 1 μl/min with Ringer's solution and dialysate samples were collected over 20 min intervals. Dialysate was collected directly into 200 μl polypropylene vials containing 5 μl of 0.05 M perchloric acid to prevent air oxidation of NE prior to analysis. The same procedure was used for in vivo experiments. The extraction efficiency (recovery) of the microdialysis probe was expressed as the percentage ratio of the concentration of NE in the collected dialysate to that in the test vial.

2.4. Analytical procedure

The HPLC system consisted of an ISCO 2350 pump, a Rheodyne 9125 injector with a 20 μl sample loop, and two LC-4B electrochemical detectors (Bioanalytical Systems, Inc., West Lafayette, IN). Separation was achieved on a Zorbax SB-C18 column (2.1 mm × 150 mm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.04 M citric acid, 0.17 mM sodium EDTA, and 2.3 mM sodium octylsulfonate. The pH of the mobile phase was adjusted to 5.0 by 6 M sodium hydroxide. The mobile phase was filtered and degassed by vacuum. Methanol, distilled prior to use, was added to give a final composition of 10% methanol (v/v). The deionized water for mobile phase preparation was distilled from the potassium permanganate solution (1 g/l) prior to use. A flow rate of 0.2 ml/min was used in all experiments.

A dual-electrode detector with glassy carbon electrodes in the series configuration was used for detection. The upstream electrode was operated at +0.5 V and the downstream electrode was operated at +0.02 V versus a Ag/AgCl reference electrode. NE was oxidized at the upstream electrode and the oxidation product detected at the downstream electrode to increase detection selectivity. Only the current at the downstream electrode was used for analytical purposes. This approach eliminates interference from compounds whose oxidation is not chemically reversible, particularly ascorbate and oxygen. A 16 μm gasket was also used in the thin-layer electrochemical cell to enhance sensitivity. The detection limit was determined to be 0.2 pg of NE in 20 μl of sample (60 pM at a S/N ratio of 3).

2.5. Microdialysis probe implantation procedure

Animals used in this study were handled in compliance with the Guiding Principles for the Care and Use of Animals, DHEW Publication No. NIH 80-23 (revised 1978, reprinted 1989; Office of Science and Health reports, DRR/NIH, Bethesda, MD, USA). Male Sprague–Dawley rats (380–450 g weight) were initially pre-anesthetized by inhalation of isoflurane and then administered ketamine/xylazine (120/15 mg/kg) as an i.m. injection to achieve full anesthesia. Anesthesia was maintained with additional i.m. injections of 50 mg/kg ketamine as needed. Artificial ventilation was performed with a constant-volume respirator using room air (Model 683 Rodent Respirator, Harvard Apparatus).

Heparin sodium (200 U/kg) was administered intravenously before probe implantation to prevent blood coagulation. With the animal in the lateral position, an incision was made between the fifth and sixth ribs on the left side, and the thoracic cavity opened using retractors. The linear microdialysis probe was implanted into the myocardial tissue of the exposed heart in the periphery of the left descending coronary artery using the attached hypodermic needle. The position of the microdialysis probe in the myocardium was fixed with a tygon ring cemented to the capillary by tissue adhesive (Vetbond) just outside of the myocardium wall (Fig. 1). The inlet and outlet tubing were passed under the skin and externalized at the rat's neck. The insertion needle was removed and the outlet tubing connected to the sample collection system. The inlet tubing was connected to the perfusion pump (CMA/100, Carnegie Medicin, Sweden) and the microdialysis probe was perfused with Ringer's solution at a flow rate of 1 μl/min.

The animal was placed in a BAS Raturn system and allowed to recover from surgery. After 1 h, collection of dialysis samples was begun to establish the basal concentration of NE in the myocardium. All experiments were conducted with a 20 min sample collection interval.

2.6. Statistical analysis

Absolute values of NE concentration in dialysate are given in ng/ml. All calculations were performed with the data expressed as percentages of mean NE value from basal levels. Basal NE concentrations were determined as the mean of at least three measurements with >5% variation obtained at the beginning of the experiment. One-way ANOVA was used to evaluate the effect of procedures on each group of animals. If a general effect was determined by ANOVA, post-hoc anal-

ysis was performed with the Scheffe or Student test with $P < 0.05$ used as the level of significance.

3. Results

3.1. Microdialysis probe characterization

Measurements in vitro showed that after 20 min of washing with the methanol, 5 mm PAN microdialysis probes exhibited recovery of NE between 50 and 70% ($62.9 \pm 0.4\%$, $n = 4$). In vitro testing of probes after 8–10 h of use in the myocardium showed no decrease in NE recovery. Recovery was found to be independent of concentration at least over the range of 0.20 mg/ml to 20.0 ng/ml.

3.2. Dual-electrode detection of NE

The use of a series configuration dual-electrode amperometric detector for improved detection of catecholamines such as norepinephrine has been reported previously [15–18]. Dual-electrode detection provides a significant enhancement in selectivity relative to single electrode detection. This results in lower detection limits. In the series configuration, the upstream electrode is used to oxidize the analyte which is then reduced at the downstream electrode. Fig. 2A shows the upstream and downstream chromatograms from a NE standard using dual-electrode detection. The oxidation of NE is chemically reversible and NE therefore gives a good signal at the downstream electrode. A typical chromatogram of a myocardial dialysate is shown in Fig. 2B. As can be seen in this figure, direct detection by oxidation at $+0.5$ V resulted in several interferences with retention times similar to NE. The selectivity was considerably higher at the downstream electrode. Only compounds whose oxidation is reversible are detected at the downstream electrode. Single electrode detection does not provide sufficient selectivity to detect basal NE in myocardium dialysis samples. NE will be significantly overestimated by single electrode detection due to co-eluting interferences. The selectivity of dual-electrode detection allowed determination of basal NE concentrations even though NE was not completely chromatographically resolved from all interferences because the co-eluting peaks do not give a response at the downstream electrode. The combination of chromatographic and electrochemical selectivity of the dual-electrode system

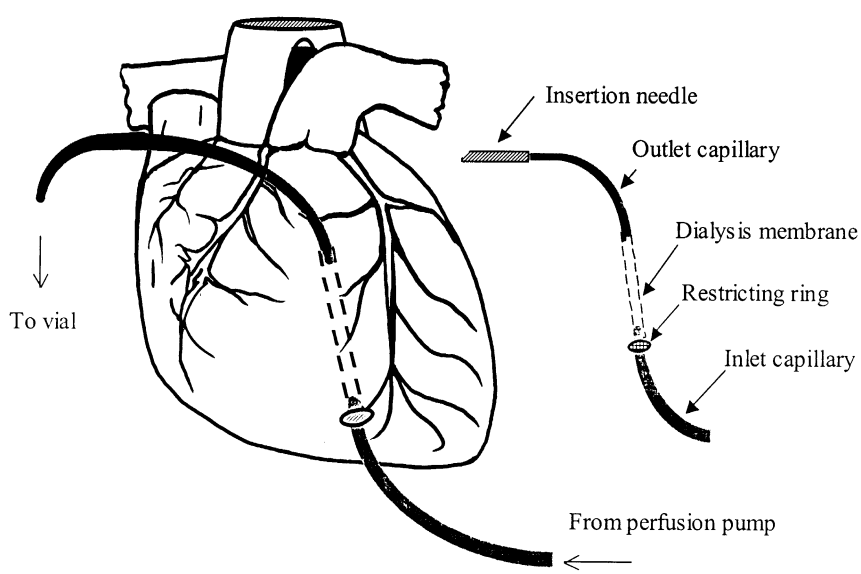

Fig. 1. Schematic representation of a microdialysis probe implanted into the rat myocardium.

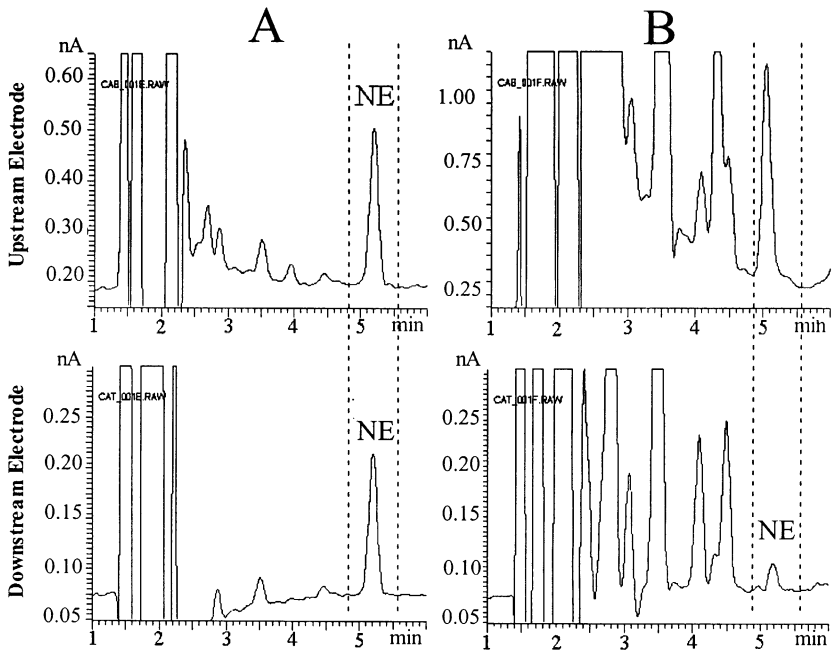

Fig. 2. Typical chromatograms of norepinephrine standard (A) and myocardial dialysate (B) using series configuration dual-electrode detection. Detection potentials: upstream electrode, +0.50 V; downstream electrode, +0.02 V.

provides the selectivity to detect basal NE in myocardial dialysis samples.

3.3. Myocardial norepinephrine in freely moving rats

The concentration of NE in the myocardial dialysate immediately after probe implantation (while the rats were still anesthetized) varied widely in the range of 0.2–0.8 ng/ml (mean 0.519 ± 0.16). This concentration decreased as the rats recovered from surgery until a steady state was reached 1.0–1.5 h after probe insertion. A steady-state dialysate basal NE concentration of 0.17 ± 0.026 ng/ml was found in awake rats.

The delivery of desipramine (0.1 mM in the perfusate) resulted in an increase in NE in the myocardium interstitial fluid. During delivery of DMI, the NE in the myocardium dialysis samples increased to $153 \pm 13\%$ of basal concentrations for five animals (Fig. 3). The influence of DMI was significant ($F(1, 31) = 17.9$; $P < 0.0002$). NE did not increase immediately upon initiating DMI delivery. The NE concentration of the first dialysis sample collected following initiation of DMI delivery was not significantly different ($106 \pm 23\%$) from the basal concentration ($F(1, 15) = 0.39$; $P > 0.54$). The NE slowly returned to basal concentrations after delivery of DMI was stopped. Sixty minutes after stopping DMI delivery (third dialysis sample), the concentration of NE was not significantly different from the basal concentration established before delivery of DMI ($111 \pm 14\%$, $P = 0.34$). Using a perfusate concentration of 1.0 mM DMI did not change the NE response relative to the delivery of 0.1 mM DMI. The NE concentration found during delivery of 1.0 mM DMI was $166 \pm 21\%$ of the basal concentration. A significant difference from the basal concentration was again observed ($F(1, 27) = 45.1$; $P < 0.0001$) however no significant difference was found between the delivery of 1.0 and 0.1 mM DMI.

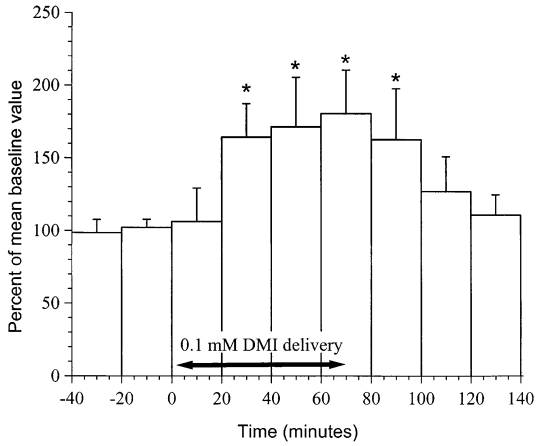

Fig. 3. Influence of DMI delivery on interstitial myocardial norepinephrine. The basal concentration of NE was determined during perfusion with ACSF, perfusion with a 0.1 mM DMI solution was begun at time = 0 and continued for 70 min (*indicates statistically different from basal concentration with $P < 0.0002$).

4. Discussion

The use of dual-electrode detection provided detection limits that allowed determination of the basal concentration of NE in the rat myocardium interstitial fluid. The improved detection limits and selectivity allowed the use of relatively small microdialysis probes. A wide range of basal NE concentrations has been previously reported using myocardial microdialysis (Table 1). The reported concentration of NE and the effect of DMI delivery depend upon the species studied, if the animal was anesthetized, and if anesthetized which anesthesia was used. These results are in good agreement with those reported in conscious rats by Cremers et al. [13]. The increase in NE observed in our study during delivery of DMI, was in good agreement with Cremers et al. rather than the much larger increases reported earlier [12,19]. One explanation for this difference could be that the microdialysis probes were not implanted in the same area of the myocardium and were not oriented similarly in all of the studies. Cremers et al. [13] reported differences in basal NE concentration in the myocardium of the right and left ventricles due to different sympathetic innervation. However, others have not observed such differences [9] using anesthetized pigs. We found no quantitative map of myocardial NE release in the literature. Another explanation could be differences in NE between the animal species used in the various studies. However, comparison of the results of Lameris et al. [12] and Mertes et al. [20] shows that an order of magnitude difference in NE has been reported for the same species. A third explanation for the difference is the use of anesthetized animals in most studies. It is possible that anesthesia decreases basal NE but does not inhibit the increase in myocardial NE release due to uptake inhibition. It has been demonstrated in rabbits [14] that the basal myocardial NE concentration is similar under pentobarbital and ketamine/xylazine anesthesia. But unlike

Table 1
Literature data on myocardial interstitial norepinephrine

Species	Anesthesia	Basal NE (ng/ml)[a]	NE after 0.1 mM DMI delivery	Reference
Pigs	Thiopentone	0.74 ± 0.02		[9]
Pigs	Thiopentone	1.49 ± 0.17[b]		[16]
Pigs	Pentobarbital	0.15 ± 0.01[b]	1.1 ± 0.15[b]	[12]
Rabbits	Pentobarbital	0.093 ± 0.014[b]		[18]
Cats	Pentobarbital	0.039 ± 0.058[c]		[8]
Cats	Pentobarbital	0.025 ± 0.012[c]	0.128 ± 0.037[c]	[15]
Cats	Pentobarbital	0.018 ± 0.003[c]	0.155 ± 0.19[c]	[19]
Rats	Chloral hydrate	25.4 ± 3.4[c]		[10]
Rats	Conscious	0.14[b]	0.21[b]	[13]

[a] Values were converted to ng/ml from the units used in the original report.
[b] Concentration based on recovery of the probe in vitro.
[c] Concentration in the dialysate.

ketamine/xylazine, pentobarbital does not suppress enhancement of NE release in response to short ischemia. The final explanation rests with the analytical methodology used to determine the NE concentration in the dialysate. Previous studies have employed a less selective single electrode scheme that this work has shown to be prone to interferences. The dual-electrode detection scheme used in our study is more selective for NE and therefore we feel provides more accurate determinations of the dialysate NE concentration.

5. Conclusions

Our results show that microdialysis sampling is useful for studying myocardial NE in conscious rats that can be used in physiological and pharmacological investigations. The use of dual-electrode detection and small linear microdialysis probes allowed the use of small, conscious animals such as rats. Local inhibition of NE reuptake by delivery of desipramine (0.1 mM) through the microdialysis probe leads to a significant increase in the dialysate NE concentration. A 10-fold increase of the DMI concentration in the perfusate did not result in a significant increase in NE release. That no difference in the myocardium NE concentration was observed using 0.1 mM and 1.0 mM DMI indicates that delivery of 0.1 mM DMI was sufficient to saturate NE reuptake inhibition.

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