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INSTRUMENTS AND TECHNIQUES

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Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice

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Abstract Epinephrine (E) and norepinephrine (NE) play a major role in regulating metabolism and cardiovascular physiology. Both are secreted in response to stress and their measurement in plasma allows the study of sympathoadrenal function. Several studies investigating sympathoadrenal physiology are conducted using mice. Review of the literature revealed that basal mouse NE and E plasma concentrations range within 4–140 nM depending on the blood sampling method. Such variability doesn't allow study comparison and may conceal catecholamine variations in response to stress. Therefore, our aim was to determine a reliable sampling method to measure mouse plasma catecholamine concentrations. Results showed that arterial catheterization is the most accurate sampling method: E and NE basal levels were similar to those found in humans (1.1±0.3 nM and 4.1±0.5 nM, respectively). Retro-orbital bleeding led to analogous results. On the contrary, decapitation was stressful for mice and consequently NE and E concentrations were high (24.6±2.7 nM and 27.3±3.8 nM, respectively). These different bleeding methods were compared in terms of their ability to detect sympathoadrenal system stimulation (cold-pressure test). With catheter and retro-orbital samplings the expected increase in NE and E levels was easily perceived. In contrast, with decapitation no significant change in E was detected. In conclusion, arterial-catheter and retro-orbital blood sampling methods appear to be the most accurate procedures for studying the sympathetic nervous system in mice in both unstressed and stressed conditions.

Keywords Blood sampling · Cold stress · Epinephrine · Mice · Norepinephrine · Retro-orbital

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Introduction

The sympathetic nervous system is one of the main regulators of blood pressure homeostasis. Since catecholamines are released from sympathetic nerve endings and the adrenal medulla, their measurement in blood is used to study sympathoadrenal function. Catecholamines are routinely quantitated in blood using radioenzymatic assay or HPLC with electrochemical detection. Stress and sympathetic activation can induce catecholamine secretion resulting in a non-specific increase in the blood catecholamine concentration. In humans, standardization of posture is essential for blood sampling because plasma catecholamine levels increase when a supine subject takes an upright position. Therefore, most procedures recommend that blood be drawn from subjects who have been resting quietly for 30 min in a recumbent position after insertion of a venous catheter. Molecular biology techniques have made it possible to generate transgenic and gene-depleted mice, which represent unique tools for studying the function of the catecholaminergic system. Some difficulties arise from the fact that mice are very small animals and, therefore, the volume of blood sample that can be obtained is small. Since one of the main interests of our laboratory is to study the sympathetic system, for which we use various mouse models, we initiated a bibliographic research on this topic and found that a variety of methods are used to collect blood from mice and that the reference plasma concentration of catecholamines varies greatly from one study to another. In these studies, the strain of mice most often used was the C57BL/6 J. Blood samples were obtained in animals

Table 1 Summary of basal catecholamine plasma concentrations in mice (C57BL/6 strain) found in various studies. Catecholamine values are expressed in nmol/l, age in weeks. (*F* Female, *M* male, *ND* no data available)

Sampling method	Anesthetic	Age/sex	NE	E	NE+E	Reference
Decapitation	ND	20/m,f	–	–	46.1±4.8	[12]
Decapitation	ND	ND/ND	59.7±7.1	79.7±6.6		[17]
Cardiac puncture	Ether	2/ND	10.3±1.4	3.1±0.7		[2]
Cardiac puncture	Asphyxia	16–32/ND	124.1±20			[14]
Cardiac puncture	Tribromoethanol	ND/ND	21.5±2.8			[5]
Tail vein	ND	12/ND	13±1.4	0.9±0.1		[11]
Retro-orbital	ND	12/m	17.7	21.8		[10]
Retro-orbital	ND	12–32/ND	13.5±0.7	13.4±0.8		[9]
Retro-orbital	Pentobarbital	9–14/ND	1.4±0.6	1.36±0.1		[3]
Retro-orbital	Pentobarbital	10/m	6.6±1.4	0.5±0.1		[15]
Carotid catheter	ND	16–32/ND			4.7±0.8	[13]
Carotid catheter	Tribromoethanol	ND	3.8±0.6			[7]
Decapitation	No	12–18/m,f	24.6±2.7	27.3±3.8		Present study
Retro-orbital	Halothane	12–18/m,f	5.8±0.8	0.4±0.1		Present study
Carotid catheter	No	12–18/m,f	4.1±0.5	1.1±0.3		Present study

of various ages (2 weeks to 1 year) by five different bleeding methods: decapitation, retro-orbital, cardiac puncture, tail vein incision, or through an intra-arterial carotid catheter. Using these different procedures norepinephrine (NE) and epinephrine (E) plasma concentrations ranged from 4 nM to 140 nM (Table 1). The lowest concentration of catecholamines (5 nM) was observed when an intra-arterial catheter was used [13] with apparently no effect of the anesthetic tribromoethanol, although E levels were not reported in this study [7]. In plasma samples obtained by tail vein incision, the concentration of catecholamines was 14 nM [11]. Retro-orbital bleeding yielded 8-fold higher values of plasma catecholamines with concentrations up to 40 nM [9, 10]. However, when mice were anesthetized with pentobarbital, catecholamine concentrations decreased up to 3–7 nM [3, 15]. Interestingly, catecholamines measurements in plasma samples obtained by cardiac puncture gave contrasting results depending on the manner by which the animal was sacrificed [2, 5, 14]. Decapitation appeared to be a stressful method with catecholamine values reaching up to 140 nM [12, 17]. Since it remains unclear whether the normal blood catecholamine concentration in adult mice is within the 5 or the 150 nM range, we compared three blood sampling protocols on age-matched C57BL/6 J mice to determine the most accurate and least stressful blood sampling method. Furthermore, we studied whether with these sampling methods a difference in catecholamines levels could be detected following an acute cold test.

Materials and methods

Animals

C57BL/6 J adult (12–18 weeks) female and male mice were used for this study. Animals were purchased from Iffa Credo (France), and were allowed to stay in the local animal facility for 2 weeks before experiments. Animals had free access to water and rodent chow. The study was approved by the local animal committee.

Blood sampling

Blood was drawn using three different sampling methods: (1) decapitation, (2) intra-arterial catheterization, and (3) retro-orbital sampling. Protocol 1: 250 μl of blood was collected by exsanguination, from non-anesthetized decapitated mice, into chilled heparin-coated tubes. Samples were inverted several times to mix blood with anticoagulant. Protocol 2: in mice anesthetized with halothane inhalation (1% to 2% in oxygen) the right carotid artery was exposed through a cervical incision and isolated by blunt dissection. PE-10 tubing filled with a solution of glucose (5%) and heparin (300 IU/ml) was inserted into the vessel. A ligature was tied around the artery, and the catheter tunneled subcutaneously to exit at the back of the neck. The skin incision was closed with surgical staples. Mice were allowed to completely recover from anesthesia for 6–7 h. Subsequently, the catheter was connected to a PE-10 tube and blood (250 μl) was directly collected in heparin-coated tubes without the use of a syringe. Mean blood pressure (MBP) and heart rate (HR) were monitored in these animals [16]. Protocol 3: mice were anesthetized for few seconds by halothane inhalation (1% to 2% in oxygen) and 250 μl of blood was drawn through the retro-orbital plexus, using ad hoc heparin-coated capillaries (Fischer Scientific, USA). Sampling was done from one eye only and the whole procedure took place in just few seconds.

Catecholamine determination

Blood samples were centrifuged at 3000 rpm for 10 min at 4°C. Subsequently, plasma was collected and quickly frozen at –70°C for 2 weeks until catecholamine measurements were performed by HPLC with amperometric detection [6] with the slight following modifications: plasma (120 μl) or standard with dihydroxybenzylamine (DHBA) (from Sigma) as internal standard was extracted on activated alumina at pH 8.6. The alumina was allowed to settle and the supernatant aspirated followed by three washes with water. The catecholamines were then eluted with 130 μl of a mixture of acetic acid 0.2 M and phosphoric acid 0.04 M (8/2, v/v) and 100 μl was injected into the chromatography system. The separation was achieved on a reversed-phase column Nucleosil 5μm C-18, 25 cm × 4.6 mm (Macherey-Nagel AG, Oensingen, Switzerland) using a 50 mM sodium acetate buffer mobile phase containing 20 mM citric acid, 0.135 mM EDTA, 1 mM dibutylamine and 3.8 mM sodium octyl sulfonate, as an ion-pairing agent, and 7% methanol at a flow rate of 0.7 ml/min. The electrochemical detector (from Antec, model Decade) was set at + 0.8 V. The following order of elution was observed: NE, E, DHBA and dopamine. The recovery is 80% and the quantification limit is 5 pg per injection. The interassay coefficients of variation were 11% for NE and 12% for E.

Cold stress

Cold stress was induced by placing mice in custom-made Plexiglas tubes immerged into ice-cold water for 15 min. In these mice, blood was sampled 7 h before the cold test and again at the end of the stress experiment either by retro-orbital or catheter procedures. In the decapitation protocol, for obvious reasons blood could be sampled only at the end of the stress experiment; however, in a similar group of mice blood was sampled by decapitation the same morning to determine basal levels. In the group of mice that underwent arterial catheterization for blood sampling, heart rate and blood pressure were also monitored before and after cold stress following the protocol described in the above section.

Statistical analysis

Results are expressed as mean ±SEM. Statistical comparisons were performed by Student's *t* test.

Results

Basal blood catecholamine levels of plasma samples obtained by decapitation were high: NE 24.6±2.7 nM and E 27.3±3.8 nM (Fig. 1A, B). In contrast, blood samples collected by the two other sampling methods showed significantly lower basal E and NE concentrations than those measured after decapitation. E levels were significantly lower in samples obtained through retro-orbital bleeding rather than arterial catheterization: 0.4±0.1 nM (n=32) vs. 1.1±0.3 nM (n=23), respectively (p<0.01) (Fig. 1A). NE concentrations were not significantly different in these two sampling methods: NE 5.8±0.8 nM vs. 4.1±0.5 nM (p=0.054) (Fig. 1B). MBP and HR values, measured in mice undergoing arterial catheterization prior to blood sampling, were within the normal range showing the absence of stress and anesthetic effect in these mice. No male to female difference in catecholamines levels was observed; therefore, results represent an average among all mice.

Cold stress

In mice undergoing blood sampling through arterial catheterization, MBP and HR were monitored. In basal conditions, before cold stress and 6–7 h after catheterization, MBP and HR values were 97±9 mmHg and 553±96 bpm, respectively. As expected, 5 min after the beginning of the cold pressor test, MBP rose by 15±9 mmHg (17±9% of basal, p<0.0001) while no change was observed on HR (525±88 bpm). MBP

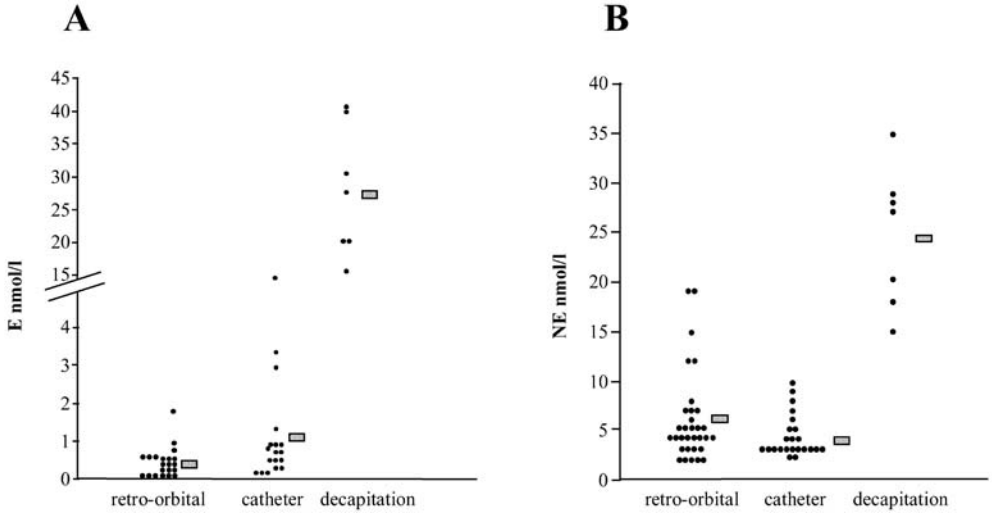

Fig. 1A, B Plasma concentrations of epinephrine (**A**) and norepinephrine (**B**) in mice. Blood was drawn by decapitation (n=7), intra-arterial catheter sampling (n=23) and by retro-orbital sampling (n=32). *Bar* indicates mean values

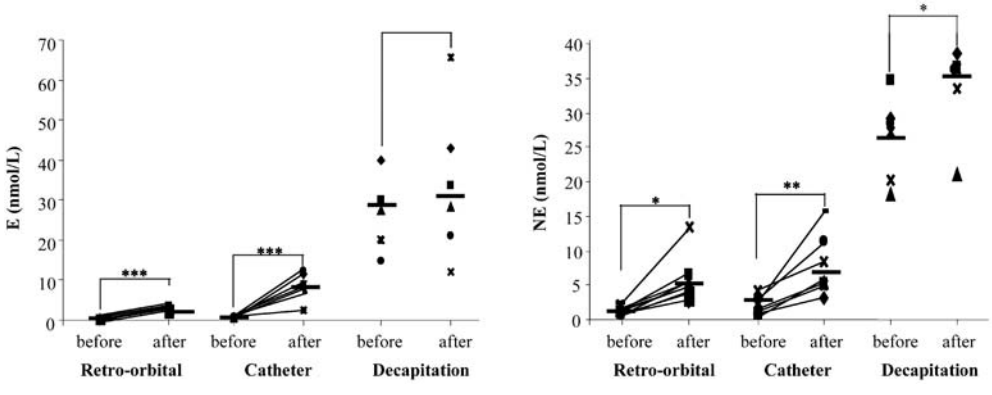

Fig. 2A, B Plasma concentrations of epinephrine and norepinephrine in mice before and after a cold stress. Blood was drawn by decapitation (n=6), intra-arterial catheter sampling (n=8) and by retro-orbital sampling (n=11). *Bar* indicates mean values. $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001

returned to baseline levels shortly after the cold stress experiment. All mice undergoing cold stress were sampled for catecholamines measurements. In blood samples collected just at the end of the cold stress with the retro-orbital method, NE concentrations rose from 2.3±0.6 nM (before cold stress) to 4.8±0.9 nM ($p<0.05$) and E from 0.1±0.02 nM to 0.9±0.15 nM ($p<0.001$) (Fig. 2). Catheter blood sampling yielded similar results to those obtained by the retro-orbital method: NE increased from 2.4±0.4 nM to 7.2±1.6 nM ($p<0.01$) and E from 0.7±0.15 nM to 7.8±1.25 nM ($p<0.001$) (Fig. 2). In contrast, in those samples obtained by decapitation, after cold stress we were not able to detect a significant increase in E levels as compared to basal conditions: 33.9±7.7 nM vs. 27.3±3.8 nM, respectively ($p=0.22$). On the contrary, in these mice a significant increase in NE plasma concentrations was observed after cold stress: 33.6±2.6 nM vs. 24.6±2.7 nM ($p<0.05$) (Fig. 2). Mice undergoing cold stress and in which blood was drawn either by catheter or retro-orbital technique were bled twice at about 1-h intervals. To ensure that double bleeding by itself was not responsible for the observed increase in catecholamines levels a control group of mice underwent serial bleeding at 1-h intervals. Catecholamines levels were determined in each sample and results showed no difference in NE or E plasma concentrations among samples.

Discussion

Our data show that the decapitation bleeding method leads to artificially very high basal plasma concentrations of E, thus preventing the detection of further catecholamine increases during an adrenosympathetic stress such as an acute cold test and despite an increase in MBP. The mechanism by which catecholamine levels are increased after decapitation could be severe peripheral vasoconstriction leading to significantly decreased perfusion of kidneys, small and large bowel, and spleen despite the highly increased perfusion pressure, followed by significant vasodilation [8]. In contrast, with retro-orbital or catheter blood sampling methods, basal catecholamine levels are similar to those found in humans, thus allowing the detection of an increase in NE and E levels such as that occurring during the course of cold stress.

Catheterization of the carotid artery remains the reference blood sampling method for catecholamine determination in mouse plasma. With arterial catheterization both NE and E concentrations appeared to be lower when compared to the other bleeding methods and are in the same range of those found by others using this methodology [7, 13]. Nevertheless, the retro-orbital bleeding method for catecholamine determination is, in our opinion and based on results presented herein, of value. In fact, with this procedure animals don't need to be sacrificed and they can be followed for a long period of time allowing the scheduling of experiments accordingly. Retro-orbital blood sampling is easily and quickly achieved as compared to carotid artery catheterization for which well trained and skilled persons are needed. Moreover, in our experience after catheterization 6–7 h is needed for complete recovery from anesthesia and to achieve normal MBP and HR values. Animals have to be sacrificed after catheterization and the great majority of catheters are no longer permeable at 24 h regardless of heparinization.

Following retro-orbital bleeding, E levels were lower than those obtained by arterial catheterization thus reflecting a non-stimulated adrenomedullary system. However, this conclusion has to be taken with care. Indeed, the halothane anesthesia used for retro-orbital bleeding, necessary for ethical and feasibility reasons, has been reported to induce a transient decrease in circulating E with a concomitant increase in NE in rats [4]. Interestingly, animals pretreated with pentobarbital and sampled by the retro-orbital method exhibited similar catecholamine concentrations to those obtained by carotid catheterization [3, 15]. However, previous studies have shown that pentobarbital anesthesia suppresses plasma catecholamine concentrations [1].

Despite the effect of anesthetic on the sympathetic system, we believe that the retro-orbital bleeding method offers an interesting alternative to arterial catheterization for catecholamine levels determination.

In conclusion, the sampling methodology and the anesthetic used in mouse models require careful consideration when interpreting data regarding the sympathetic nervous system.

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