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Acute isoproterenol induces anxiety-like behavior in rats and increases plasma content of extracellular vesicles

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HIGHLIGHTS

- Isoproterenol (5 mg kg^{-1}) increases HR and induces anxiety-like behavior in rats.
- The same treatment increases blood content of extracellular vesicles (EVs).
- Stress markers (corticosterone and EV Hsp70 content) are not modified.
- Peripheral signals via EVs can reach the CNS and modulate anxiety.

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ABSTRACT

Several clinical observations have demonstrated a link between heart rate and anxiety or panic disorders. In these patients, β-adrenergic receptor function was altered. This prompted us to investigate whether the β-adrenergic receptor agonist isoproterenol, at a dose that stimulates peripheral β-adrenergic system but has no effects at the central nervous system, can induce anxiety-like behavior in rats. Moreover, some possible messengers involved in the peripheral to brain communication were investigated. Our results showed that isoproterenol (5 mg kg^{-1} i.p.) increased heart rate, evoked anxiety-like behavior, did not result in motor impairments and increased extracellular vesicle content in the blood. Plasma corticosterone level was unmodified as well as vesicular Hsp70 content. Vesicular miR-208 was also unmodified indicating a source of increased extracellular vesicles different from cardiomyocytes. We can hypothesize that peripheral extracellular vesicles might contribute to the β-adrenergic receptor-evoked anxiety-like behavior, acting as peripheral signals in modulating the mental state.

1. Introduction

Lang described the so called James and Lange theory of emotion by saying that emotion was the feeling of bodily changes, which follow the perception of an exciting event, hence bodily and behavioral responses in emotion were prior events [1]. Cannon criticized the James and Lange theory by observing that the visceral processes are extraordinarily undemonstrative, and artificial visceral stimulation (as provided by a shot of adrenalin) does not produce strong emotions. Moreover, the visceral changes noted upon presentation of an external stimulus are slower than the emotional reaction [2]. Even if Cannon's criticism is generally accepted, several experimental and clinical observations

Abbreviations: EV, extracellular vesicle; miR, microRNA; CNS, central nervous system; HR, heart rate.

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have demonstrated a link between heart rate and anxiety or panic disorders [3–7]. In particular, β-adrenergic receptor function seems to be altered in patients with anxiety disorders and decreased β-adrenergic receptor responsiveness has been reported in patients with panic disorder [8,9].

A lot of signal molecules released by peripheral cells into blood are able to carry messages to the central nervous system. Recently, extracellular vesicles (EVs) have been recognized as new actors able to carry multiple molecular messengers at a long-distance [10]. Among EVs, exosomes have peculiar characteristics; they originate from intracellular endosomal compartment, have a diameter of 30–100 nm and express specific markers such as Tsg-101 on their limiting membrane which allows their isolation [11]. Moreover, many recent literature data suggest the ability of exosomes to cross the blood–brain barrier [12].

In the present study, the effect of the β-adrenergic receptor agonist isoproterenol, at a dose [13] that increases heart rate (HR) but has no effects at the central nervous system (CNS), has been evaluated on anxiety-like behavior of rats. In particular, the association between HR increase and anxiety was investigated by means of the open field test. Furthermore, plasma content of EVs was evaluated by means of the specific exosome marker Tsg-101. To investigate a possible stress condition induced by isoproterenol, with consequent involvement of glucocorticoids in the anxiety-like behavior, plasma corticosterone level was evaluated. An increase of heat shock protein 70 (Hsp70) plasma level has also been associated with a stress condition [14,15]; moreover, Hsp70 is mainly released inside EVs [16,17]. Therefore, EV content of Hsp70 was detected. Finally, the EVs have been characterized for their content in miR-208, specifically expressed in cardiac myocytes, to study this possible cellular source of EVs [18,19].

2. Materials and methods

2.1. Animals

Adult male pathogen-free Sprague Dawley rats (Harlan-Nossan, Milan, Italy), weighing 185–200 g and maintained in the SPF Animal Interdepartmental Service Unit at University of Modena and Reggio Emilia, Modena, Italy, were used. The rats were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (lights on 7 AM–7 PM). Food and water were freely available. All experimental procedures were in accordance with the European Community (Directive 86/609/EC) and with the endorsement of the Animal Ethics Committee of the University of Modena and Reggio Emilia (codice A96 of 17 December 2009) and of Italian Ministry of Health (04 February 2010). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Experimental groups

Two groups of 30 rats were used: one received saline and the other was treated with isoproterenol. Isoproterenol hydrochloride (Sigma Aldrich, Italy) was dissolved in saline and intraperitoneally (i.p.) injected at the dose of 5 mg kg^{-1} ml^{-1}. As reported in literature [20], this dose does not lead to myocardial necrosis or histological changes. From each group, 10 animals were used for open field test, 10 for spontaneous motor activity testing and 10 for ECG record and plasma sample collection.

2.3. ECG

ECG was recorded for 5 min on rats anesthetized with chloral hydrate (200 mg kg^{-1}, i.p.), 40 min after saline or isoproterenol injection. Heart rate was recorded by means of a transducer (P23 Db, Statham, Oxnard, CA) coupled with a polygraph (Battaglia-Rangoni, produced by Mortara Rangoni Europe, Bologna, Italy). Heart rate was determined by counting the number of QRS complexes in a 10-second period (the typical length of a standard ECG) and then multiplying the number by 6. Rhythm was also evaluated by taking into account the R to R interval in the same 10-second period.

2.4. Open field test

The open field exploration test is a well-validated assay to analyze both locomotion and mood-related behavior. Speed and total distance moved provide information about the general exploratory/locomotor activity of rats. It is a matter of particular interest which zone of the open field the rats prefer. Anxiety-like behavior is reflected by staying in the corners and in the border area, whereas bold behavior is indicated by exploration of the center of the arena. The open field arena consisted of a square box (100 × 100 cm) made of gray PVC and located in an illuminated room (12 lx as measured at the bottom of the open field boxes). A square of 40 × 40 cm in the middle of open field box was designated as the inner zone. Open field behavior (distance in cm, speed in cm s^{-1}, time spent in the outer and inner zones) was measured via a computerized animal observation system (Smart system version 2.5) that was connected to a camera mounted on the ceiling above the open field box. All animals, in their cages, were brought into the testing room for 60 min before the start of session and then they were placed in the center of the arena and tested in open field box for 10 min, 45 min after saline or isoproterenol (5 mg kg^{-1}) i.p. injection.

2.5. Spontaneous motor activity testing

Rat locomotor activity was measured through activity cages (Letica LE886) with automatic counting of movements (LE3806) across the bars on the cage floor. On the morning of testing, rats were transferred into the test room at least 2 h prior to drug administration. Forty minutes after intraperitoneal administration of isoproterenol (5 mg kg^{-1}) or saline, animals were transferred individually to Letica activity cages and after 5 min latency period, activity counts were recorded for 30 min at 10, 20 and 30 min. A saline treated rat and an isoproterenol treated rat were paired tested in each locomotor activity testing session. The cage floor was wiped clean with 75% alcohol solution between the tests of each animal.

2.6. Plasma sample collection

Animals were anesthetized with chloral hydrate (200 mg kg^{-1}, i.p.) 45 min after i.p. injection of isoproterenol (5 mg kg^{-1}) or saline, and blood was drawn by cardiac puncture. Blood samples were collected into Vacutainer K2E (5.4 mg) tubes and centrifuged within 2 h at 800 *g* at 4 °C for 10 min to obtain plasma which was further centrifuged at 13,000 *g* at 4 °C for 20 min. Supernatants (2.5 ml for each animal) were collected and stored at −80 °C.

2.7. Isolation of circulating EVs

Plasma samples (2 ml for each animal) were diluted to 10 ml with cold PBS, and centrifuged at 10,000 *g* for 20 min at 4 °C to remove large debris. Subsequently, the supernatants were further centrifuged for 20 min at 18,000–20,000 *g*. The resulting supernatants were filtered through a 0.22 μm filter and then EVs were pelleted by ultracentrifugation at 110,000 *g* for 70 min. The EV pellets were washed in 13 ml PBS, pelleted again and resuspended in 50 μl PBS. Twenty microliters of EVs were used for immunoblotting [21,22].

2.8. Western blotting analysis

For electrophoresis, samples were mixed with Laemmli sample buffer 4× (1:4 ratio) and loaded onto 12% SDS-PAGE gels. Subsequently, proteins were blotted to a nitrocellulose membrane (GE Healthcare). Primary antibodies used were versus Tsg-101 (1:2000 dilution, clone