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Original article

Sexually diergic, dose-dependent hypothalamic–pituitary–adrenal axis responses to nicotine in a dynamic *in vitro* perfusion system

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ABSTRACT

Introduction: The hypothalamic–pituitary–adrenal cortical (HPA) axis modulates physiological responses to stress. We previously reported sexually diergic, dose-dependent HPA responses *in vivo* following nicotine administration: Male rats had greater arginine vasopressin (AVP) responses than females, and female rats had greater adrenocorticotropic hormone (ACTH) and corticosterone (CORT) responses than males. The goal of the present study was to further investigate sexually diergic, dose-dependent HPA responses following nicotine addition to an *in vitro* model of the HPA axis, so that hormone output could be determined at each level of the axis. **Methods:** Hypothalami, pituitaries, and adrenal glands were harvested from male and female rats. One-half hypothalamus, one-half pituitary, and one adrenal gland were placed individually into three jacketed tissue baths connected by tubing and perfused in series with physiological medium. Sampling ports between tissue baths were used to collect buffer before and after addition of various doses of nicotine, for measurement of AVP and corticotropin-releasing hormone (CRH) from the hypothalamus bath, ACTH from the pituitary bath, and CORT from the adrenal bath. Hormones were measured by highly specific immunoassays. **Results:** Stable temperatures, flow rates, pH, and hormone baselines were achieved in the *in vitro* system. Consistent with our *in vivo* and earlier *in vitro* studies, nicotine added to the hypothalamus tissue bath significantly increased HPA responses in a sex- and dose-dependent manner: Males had greater AVP responses than did females, and females had greater CRH responses than did males. Sexually diergic ACTH and CORT responses were less apparent and were higher in females. **Discussion:** Our *in vitro* system accurately models *in vivo* HPA responses to nicotine in both sexes and thus represents a reliable method for investigating the effects of nicotine on components of the HPA axis. These studies may be pertinent to understanding the biological differences to nicotine between men and women smokers.

1. Introduction

Cigarette smoking is a major worldwide health concern for both men and women (Benowitz, 2008; Rose, 2008). Nicotine (NIC) administration via various routes increases stress hormone concentrations in both humans (al'Absi, 2006; Mendelson, Sholar, Goletiani, Siegel, & Mello, 2005; Mendelson, Goletiani, Sholar, Siegel, & Mello, 2008; Pomerleau et al., 2004; Rohleder & Kirschbaum, 2006) and rodents (Cam & Bassett, 1983; Cam, Bassett, & Cairncross, 1979; Fu, Matta, Valentine, & Sharp, 1997; Matta, Beyer, McAllen, & Sharp, 1987; Yu, Chen, Zhao, Matta, & Sharp, 2008). Biological sex appears to be an important factor underlying stress hormone sensitivity following NIC (Faraday, Blakeman, & Grunberg, 2005). Therefore, basic studies of sex differences in stress hormone responses to NIC are relevant to the understanding of biological differences between men and women smokers.

The hypothalamic–pituitary–adrenal (HPA) axis is a three-gland component of the endocrine system that modulates biological responses to acute and chronic stress (Bugajski, Gadek-Michalska, Borycz, & Glod, 1999). Immediately following stress, HPA axis activity increases, initiated by the release of corticotropin-releasing hormone (CRH) from neurons of the paraventricular nuclei of the hypothalamus. CRH, in turn, stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary, and ACTH stimulates corticosterone (CORT) release from the adrenal cortex. Arginine vasopressin (AVP), released from neurons of the paraventricular and supraoptic nuclei of the hypothalamus, acts as a potent secretagogue of ACTH release, potentiating the CRH-driven stress response (Aguilera, Subburaju, Young, & Chen, 2008; Antoni, 1993; Engelmann, Landgraf, & Wotjak, 2004; Surget & Belzung, 2008; Volpi, Rabadan-Diehl, & Aguilera, 2004).

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Several neurotransmitters, including acetylcholine (ACh), modulate HPA axis activity (Rhodes, O'Toole, Wright, Czambel, & Rubin, 2001; Rhodes & Rubin, 1999; Whitnall, 1993) and are released in response to NIC (Albuquerque et al., 2000; Benowitz, 2008; Paterson & Nordberg, 2000). NIC, the main psychoactive component of cigarettes, has been shown to activate the HPA axis in a dose-dependent manner (Mendelson et al., 2005; Porcu et al., 2003; Rhodes, O'Toole, Czambel, & Rubin, 2001; Rhodes, Kennell, Belz, Czambel, & Rubin, 2004). Reports of sex differences in HPA axis responses to NIC are relatively few (e.g., (Faraday et al., 2005; Grota, Bienen, & Felten, 1997; Moidel, Belz, Czambel, Rubin, & Rhodes, 2006; Pogun & Yararbas, 2009; Rhodes et al., 2004). Our previous *in vivo* studies indicate that NIC activates the HPA axis in a sexually diergic manner, males having significantly greater plasma AVP responses than females, and females having significantly greater plasma ACTH and CORT responses than males (Rhodes, O'Toole, Czambel et al., 2001).

We previously reported that, in a dynamic perfusion system of HPA tissues, NIC also activates the HPA axis in a sexually diergic manner (Moidel et al., 2006). However, only one NIC dose was used in the previous study and AVP was not measured. The purposes of the present study were (1) to further validate this *in vitro* model with improved system components and physiological parameters; (2) to determine sexually diergic CRH, AVP, ACTH, and CORT responses to cholinergic stimulation by several doses of NIC; and (3) to compare the results with previously determined *in vivo* and *in vitro* hormone responses to NIC stimulation.

2. Methods

2.1. Animals

All animal procedures were approved by the Saint Vincent College Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines for proper animal care. Eight-week-old male and female Sprague–Dawley rats weighing 200–225 g were obtained from Taconic Farms, Inc. (Germantown, NY, USA). All animals were housed doubly in a well-ventilated, temperature- and humidity-controlled environment (22–25 °C, 50–75% humidity) and were maintained on a 12-h light–dark cycle (lights on at 0800 h) with food and water available *ad libitum*.

2.2. Tissue isolation

Rats were euthanized by isoflurane (Baxter, Deerfield, IL, USA) inhalation prior to decapitation, methods consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association. Immediately following decapitation, the hypothalamus, pituitary gland, and adrenal glands were collected. The hypothalamus was isolated by the block method (Hatton, Doran, Salm, & Tweedle, 1980). The pituitary was collected from the sella turcica after severing the optic nerves and infundibulum and removing the brain from the skull. The hypothalamus and pituitary were bisected prior to weighing. Adrenal glands were removed by ventral approach and were cleared of adipose tissue. Tissue isolation time was approximately 5 min for each animal.

Each hypothalamic and pituitary tissue-half and each adrenal gland was weighed and placed individually in beakers containing modified Bradbury tissue culture medium (pH 7.4) at 37 °C (Bradbury, Burden, Hillhouse, & Jones, 1974; Garrido, Manzanares, & Fuentes, 1999). The culture medium contained (in mM) 126 NaCl, 6 KCl, 0.88 $MgSO_4$, 1 Na_2HPO_4, 22 $NaHCO_3$, 1.45 $CaCl_2$, 11 glucose, and 0.05 ascorbic acid (all reagents from Sigma, St. Louis, MO, USA). The tissues were immediately enclosed in 100 mesh, 20 mm (3/4") circular, stainless-steel screens (Small Parts, Inc., Miami Lakes, FL, USA) that were folded in half and held together with 9 mm stainless-steel wound clips (World Precision Instruments, Sarasota, FL, USA). These were transferred to 10-ml jacketed tissue baths (World Precision Instruments, Sarasota, FL, USA) comprising the *in vitro* perfusion system (Fig. 1). The hypothalamus baths were connected to the source buffer flask via Tygon flexible plastic tubing (3.2 mm [1/8"] I.D.; 6.4 mm [1/4"] O.D.; 1.6 mm [1/16"] wall thickness). The jacketed tissue baths and a terminal siphon regulator were connected to each other by Tygon flexible plastic tubing (3.2 mm [1/8"] I.D.; 4.8 mm [3/16"] O.D.; 0.8 mm [1/32"] wall thickness) (Cole-Palmer, Vernon Hills, IL, USA) with Luer connectors (Cole-Palmer, Vernon Hills, IL, USA) and 3-way stopcocks (World Precision Instruments, Sarasota, FL, USA), to enable the connection of sampling ports composed of 3.2 mm Tygon tubing plus Luer connectors (Cole-Palmer, Vernon Hills, IL, USA). The interior surface of each 10-ml jacketed tissue bath was coated with silicone lubricant (Dow Corning Corp., Midland, MI, USA) to prevent ACTH from binding to the glass. Each jacketed tissue bath was oxygenated via Tygon tubing (3.2 mm [1/8"] I.D.; 6.4 mm [1/4"] O.D.; [1/16"] wall thickness) connected to a polycarbonate multi-port manifold (Cole-Palmer, Vernon Hills, IL, USA). The flow rate between baths was regulated by the speed of the perfusion pump (Minipuls 3, Gilson), by changing the height of the tissue baths in relation to one another, and by the terminal siphon regulator which was connected to the terminal end of the adrenal jacketed tissue bath via Tygon tubing. The terminal siphon regulator ensured a steady flow rate out of the adrenal jacketed tissue bath, and thus through the entire *in vitro* system. A Bechman Centerline II circulating water bath was used to circulate water (38 °C) through the jackets surrounding the tissue baths. The tissues were equilibrated by perfusion of 37 °C culture medium for at least 30 min before experiments were initiated.

2.3. In vitro system

Modified Bradbury culture medium warmed (49–60 °C; depending on ambient room temperature, flow rate through the system, and tissue bath temperatures) and buffered (pH 7.3–7.5) at the 1 l source flask was perfused through the system by a perfusion pump (Gilson Minipuls 3, Middleton WI, USA) (Fig. 1). A 1 l reserve flask containing warmed culture medium was used to replenish the medium of the source flask as necessary during the experiments. Each jacketed tissue bath was gassed with 95% O_2, 5% CO_2 and had a total medium volume of approximately 6 ml. Oxygenation flow levels were controlled in each jacketed tissue bath via Keck Ramp Clamp Tubing Clamps (Cole-Parmer, Vernon Hills, IL, USA). Including volume loss from the system during sampling, flow rates of the medium through the system during the experiments ranged between 1.1 and 1.9 ml/min. Temperatures ranged between 36.1 and 38.4 °C in hypothalamus baths, 37.1°–38.5 °C in pituitary baths, and 36.2°–38.9 °C in adrenal baths. At the end of each experiment, the tissues were exposed to 60 mM KCl for 10 min to test tissue responsiveness and viability to membrane depolarization (Gao, Zhang, & Ju, 2000). Temperature and pH of buffer in the source flask, the reserve flask, and each jacketed tissue bath were continuously measured throughout each experiment with alcohol thermometers (Allegiance, McGraw Park, IL, USA), individual digital thermometers (VWR, West Chester, PA, USA) and pH electrodes and meters (Radiometer PHM92, Cedex, France). Due to heating, the pH of the Modified Bradbury buffer gradually rose throughout experimentation. The pH of the buffer was thus regulated via drop-wise additions of 1 N HCl.

2.4. Sampling for stability of baseline hormone concentrations

At the beginning of each experiment, baseline culture medium samples were taken from each of the sampling ports at 0, 5, 10, 15, and 20 min following the 30 min tissue equilibration for determination of CRH, AVP, ACTH, and CORT concentrations. Additional baseline culture medium samples were taken prior to each NIC administration. Following NIC stimulations at 70, 110 and 150 min, hormone