Estrogen provokes the depressant effect of chronic nicotine on
vagally-mediated reflex chronotropism in female rats

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Estrogen exacerbates nicotine-evoked baroreflex dysfunction

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Abbreviations: BP, blood pressure; HR, heart rate; MAP, mean arterial pressure; BRS, baroreflex sensitivity; OVX, ovariectomy; SO, sham-operated; E2, estradiol; PE, phenylephrine; SNP, sodium nitroprusside.

Section assignment: Cardiovascular
Abstract

We recently reported that acute nicotine impairs reflex tachycardic activity in estrogen-depleted but not repleted female rats, suggesting a restraining influence for estrogen against the nicotine effect. In this contribution, we tested whether the baroreflex protective effect of estrogen can be replicated when nicotine was administered chronically. We also report on the dose dependency and autonomic modulation of the nicotine-baroreflex interaction. The effects of nicotine (0.5, 1, or 2 mg/kg/day for 14 days) on baroreflex curves relating changes in heart rate to increases (phenylephrine, PE) or decreases (sodium nitroprusside, SNP) in blood pressure were evaluated in sham-operated (SO), ovariectomized (OVX), and estrogen-replaced OVX (OVXE2) rats. Slopes of the curves were taken as a measure of baroreflex sensitivity (BRS<sub>PE</sub> and BRS<sub>SNP</sub>). In SO rats, both reflex bradycardic and tachycardic responses were attenuated by nicotine in a dose-related fashion. In nicotine-treated rats, blockade of β-adrenergic (propranolol) but not muscarinic (atropine) receptors caused additional reductions in reflex chronotropic responses, implying that nicotine selectively impairs reflex vagal activity. OVX selectively decreased BRS<sub>PE</sub> but not BRS<sub>SNP</sub> and abolished the nicotine-induced impairment of either response. These effects of OVX were reversed after treatment with estrogen or the estrogen receptor modulator raloxifene. In atropine-treated rats, comparable BRS values were demonstrated in all rat preparations regardless of the estrogen or nicotine milieu. Collectively, the inhibition of vagal activity accounts for the depressant effect of chronic nicotine on baroreflex activity. Further, contrary to its acute effects, the baroreflex attenuating effect of chronic nicotine is exacerbated by estrogen.
Tobacco smoking is a major risk factor for cardiovascular diseases including hypertension, atherosclerosis, coronary heart disease, acute myocardial infarction, and sudden cardiac death (Barnoya and Glantz, 2005; Bullen, 2008). The nicotine content of tobacco smoke is highly blamed for the deleterious cardiovascular consequences of cigarette smoking (Balakumar and Kaur, 2009). The increased cardiovascular risk is also seen in people using nicotine replacement therapy to facilitate tobacco cessation (Schnoll and Patterson, 2009). The adverse effects of nicotine have been attributed largely to increased activity of the sympathetic nervous system (Narkiewicz et al., 1998). Arterial baroreceptor dysfunction is another important mechanism that contributes to increased vulnerability of smokers to cardiovascular risk (Mancia et al., 1997). Nicotine diminishes the baroreflex gain through direct interaction with central mechanisms integrating the baroreceptor input into autonomic responses (Ashworth-Preece et al., 1998) or via reducing arterial compliance and stretch receptor responsiveness (Giannattasio et al., 1994). Nicotine also modifies the effector responsiveness to reflex autonomic modulation (Niedermaier et al., 1993).

Recent experimental reports from our laboratory showed that the interaction of acutely administered nicotine with reflex chronotropic responses depended on the animal sex, hormonal milieu, and nature of the baroreflex heart rate (HR) response (El-Mas et al., 2011a, 2011c). In male rats, nicotine impaired the baroreceptor-mediated control of reflex tachycardia but not bradycardia through a mechanism that involved disruption of adenosine A2A receptor-mediated facilitation of reflex cardiac sympathoexcitation (El-Mas et al., 2011a). The depressant effect of nicotine on reflex tachycardia is also demonstrated in female rats with depleted (OVX or diestrus rats) but not physiological (proestrus rats or estrogen-replaced OVX rats) estrogen levels, suggesting a protective effect for estrogen against the nicotine-baroreflex interaction (El-Mas et al., 2011c). Pharmacological evidence also implicated central neural pools of estrogen receptors...
in the protection offered by estrogen against nicotine-induced baroreceptor dysfunction in estrogen-depleted female rats (El-Mas et al., 2011c). Clinically, the attenuation of compensatory sympathoexcitation by nicotine may have detrimental consequences in conditions such as hypothalamic defense response, posture changes, and ventricular rhythms (Hayano et al., 1990; Duan et al., 1999; Smith et al., 1999).

Because nicotine was given acutely in our previous studies (El-Mas et al., 2011a, 2011c), the issue whether the preferential depressant action of nicotine on reflex tachycardia could be replicated when nicotine is administered on chronic basis has not been explored. Therefore, this study reports on the dose-dependency of chronic nicotine on cardiovascular and baroreflex functions in conscious female rats. More importantly, the estrogenic and autonomic modulation of the nicotine-baroreflex interaction was also investigated. The results showed that unlike its acute effects (i) both reflex tachycardic and bradycardic responses were attenuated by chronic nicotine, (ii) estrogen exacerbated, rather than protected against, the nicotine-evoked baroreflex dysfunction, and (iii) the inhibition of cardiac vagal activity accounted for the baroreflex depressant effect of nicotine.
Materials and methods

Female Wistar rats (200-250 g, Faculty of Pharmacy animal facility, Alexandria, Egypt) were used. All experiments were approved by the institutional animal care and use committee and carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Intravascular cannulation. The method described in our previous studies (El-Mas and Abdel-Rahman, 1993; El-Mas et al., 2011a) was adopted. Briefly, rats were anesthetized with thiopental (50 mg/kg i.p.). Catheters (each consisted of a 5-cm polyethylene-10 tubing bonded to a 15-cm polyethylene-50 tubing) were placed in the abdominal aorta and vena cava via the femoral artery and vein for measurement of blood pressure (BP) and intravenous administration of drugs, respectively. The polyethylene-10 portion was used for the intravascular segment of the catheter. The arterial catheter was connected to a blood pressure transducer (Model P23XL, Astro-Med, Inc., West Warwick, RI, USA) that was attached through MLAC11 Grass adapter cable to a computerized data acquisition system with LabChart-7 pro software (Power Lab 4/35, model ML866/P, AD Instruments, Bella Vista, Australia). HR was computed from BP waveforms and displayed on another channel of the recording system.

Finally, catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. The catheters were flushed with heparin (0.2 ml, 100 U/ml) and plugged by stainless steel pins and incisions were closed by surgical clips. Each rat received an intramuscular injection of 60,000 U of penicillin G benzathine and penicillin G procaine (Penicid) and housed in a separate cage. Experiments started 2 days later in conscious freely moving rats. This time period has been shown adequate for recovery from surgery and restoration of normal rat activity (El-Mas and Abdel-Rahman, 1993; El-Mas et al., 2011a).
Ovariectomy. Bilateral ovariectomy was performed 14 days before experimentation, i.e. 12 days before intravascular cannulation, as described in our previous studies (El-Mas and Abdel-Rahman, 1998, 2001).

Measurement of plasma cotinine. A blood sample was drawn from the arterial line 2-3 hr after the last dose of nicotine (i.e. day 14) and centrifuged at 800 g for 10 min. The plasma was aspirated and stored at -20°C till analyzed for cotinine levels, the main metabolite of nicotine, by the chemiluminescent immunoassays (EURO/DPC Ltd, DPC Scandinavia, Mölndal, Denmark) as reported elsewhere (El-Mas et al., 2011a).

Protocols and experimental groups

Cardiovascular effects of chronic nicotine in female rats. In this experiment, we investigated the effect of chronic nicotine on cardiovascular and baroreflex-HR responses and the modulation of these responses by cardiac vagal and sympathetic activities in conscious female rats. Four groups of female rats (n=6-8 each) were used to determine the effects of i.p. nicotine (0.5, 1, or 2 mg/kg/day for 14 days) or equal volume of saline on systolic BP, HR, and reflex chronotropic responses. Tail cuff measurements of systolic BP were performed before the start of the nicotine or saline regimen (baseline values) and then 5, 10, and 14 days thereafter. BP was measured 2-3 hr after nicotine or saline administration. A computerized data acquisition system with LabChart-7 pro software (Power Lab 4/30, model ML866/P, AD Instruments, Bella Vista, Australia) was employed for data recording as in our previous studies (El-Mas et al., 2011a, 2011c). HR was computed from BP waveforms and displayed on another channel of the recording system.

For baroreflex testing, rats were instrumented for direct BP and HR measurements 2 days before experimentation (i.e. 12 days after nicotine administration). On the experiment day, the arterial catheter was connected to the pressure transducer and Power Lab data acquisition system for measurement of BP and HR as detailed above. A period of 30 min was allowed at the
beginning of the experiment to permit hemodynamic stabilization. Baroreflex sensitivity was assessed by the vasoactive (Oxford) method (El-Mas and Abdel-Rahman, 1998; Saleh et al., 2000; El-Mas et al., 2011a), which measures bradycardic or tachycardic responses to reciprocal peripherally-mediated BP changes evoked by bolus i.v. injections of randomized doses of PE or SNP (1-16 μg/kg, every 5 min). PE and SNP were dissolved in saline and the injection volume was kept constant at 0.05 ml/100 g body weight with a flush volume of approximately 0.1 ml saline. The mean arterial pressure (MAP, computed via integration of the area under the pulse pressure curve using the LabChart software) and HR values before and after PE or SNP administration were determined and peak changes in both variables (ΔMAP and ΔHR) were used for the construction of the baroreflex curves (El-Mas and Abdel-Rahman, 1998; El-Mas et al., 2011a). Slopes of the regression line (BRS<sub>PE</sub> and BRS<sub>SNP</sub>) were taken as an index of baroreflex sensitivity.

To determine the role of cardiac autonomic control in the nicotine-baroreflex interaction, baroreflex curves of PE and SNP were re-established in rats (the saline and nicotine 2 mg/kg/day groups) 10 min after i.v. administration of 1 mg/kg dose of atropine (muscarinic blocker) or propranolol (β-adrenergic blocker) (El-Mas et al., 2002b, 2011a). Each rat in a particular group was employed in two experiments (2 and 4 days after intravascular cannulation, i.e. 14 and 16 days after nicotine or saline treatment) to test the effect of atropine or propranolol. In the first experiment, approximately 50% of rats in a given group (saline or nicotine 2 mg/kg/day) received atropine and the other 50% received propranolol. In the second experiment, the administration of atropine and propranolol was crossed over. Comparison of the BRS before and after atropine or propranolol would allow a proper assessment of the relative contributions of the vagal and sympathetic autonomic components to reflex HR responses (El-Mas et al., 2002a, 2002b, 2011a).
One more group of rats (n=7) was employed to test the effect of total autonomic blockade with atropine and propranolol on reflex chronotropic activity. HR responses to PE and SNP were evaluated before and after simultaneous i.v. administration of atropine and propranolol (1 mg/kg each).

**Estrogenic modulation of the autonomic and baroreflex effects of nicotine.** This experiment tested the hypothesis that the inhibition of the facilitatory baroreflex and autonomic effects of estrogen mediates the depressant effect of nicotine on reflex chronotropic responses in female rats. Four groups of rats (n=6-8 each) were employed to determine the effect of i.p. nicotine (2 mg/kg/day for 14 days) or saline on baroreflex gain in ovariectomized rats treated with (OVXE2) or without estrogen (OVX). For estrogen replacement, OVX rats received a daily s.c. dose of 17β-estradiol of 50 μg/kg for 5 consecutive days starting from the 9th day after OVX. The last dose of 17β-estradiol was administered 24 hr before the experiment. This dose regimen of 17β-estradiol has been shown in our previous studies to produce physiological estrogen levels (El-Mas et al., 2009, 2011b). Rats in the OVX group received the vehicle (sesame oil) instead of 17β-estradiol.

All rats were instrumented for BP and HR measurements 2 days before experimentation (i.e. 12 days after OVX). On the experiment day (day 14 after OVX), the arterial catheter was connected to the pressure transducer and Power Lab data acquisition system for measurement of BP and HR and baroreflex curves of PE and SNP were generated as described above. Afterwards, atropine (1 mg/kg) was administered intravenously and 10 min later the baroreflex curves of PE and SNP were re-established. The effect of atropine was tested because results of preceding experiments implicated vagal cardiomotor dysfunction in the baroreflex depressant action of nicotine.

The possibility that the selective estrogen receptor modulator raloxifene mimics the effect of estrogen on the nicotine-baroreflex interaction in OVX rats was investigated. Two groups of OVX rats (n=7 each) were used and given raloxifene (10 mg/kg/day s.c.) alone or combined with
nicotine (2 mg/kg/day i.p.). The nicotine regimen continued for 14 days after OVX whereas raloxifene was given for 5 days starting from day 9 of OVX. On day 14, baroreflex curves of PE and SNP were constructed.

To further support the importance of estrogen in baroreflex dysfunction caused by nicotine, four groups of female rats (2 proestrus and 2 diestrus, n=6 each) were used to determine the influence of the phase of the estrus cycle on the nicotine-baroreflex interaction. Diestrus rats exhibit significantly lower estrogen levels compared with proestrus rats (El-Mas et al., 2011b). The phase of the cycle was identified through microscopic examination of vaginal smears. Two groups of rats (one proestrus and one diestrus) received nicotine (2 mg/kg/day) for 2 weeks. The other two groups received saline and served as controls. Reflex chronotropic responses to PE and SNP were assessed as described earlier.

**Drugs.** Phenylephrine hydrochloride, sodium nitroprusside, 17β-estradiol, raloxifene (Sigma Chemical Co., St. Louis, MO, U.S.A.), nicotine (Merck Schuchardt OHG, Hohenbrunn, Germany), thiopental (Thiopental, Biochemie GmbH, Vienna, Austria), povidone-iodine solution (Betadine, Nile Pharmaceutical Co., Cairo, Egypt) and Penicid (Cid Pharmaceutical Co., Cairo, Egypt) were purchased from commercial vendors.

**Statistical analysis.** Values are expressed as means±SEM. The relationship between changes in MAP evoked by PE or SNP and associated reciprocal changes in HR was assessed by regression analysis for individual animals as described in our previous studies (El-Mas et al., 2002b, 2011a). The regression coefficient (slope of the regression line, $BRS_{PE}$ and $BRS_{SNP}$) expressed as beats/min/mmHg was taken as an index of baroreflex responsiveness. Analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc analysis was used for two-way repeated measures or multiple comparisons with the level of significance set at $P<0.05$. 
Results

Effect of chronic nicotine on baseline systolic BP and HR

The tail-cuff measurements showed that compared with saline-treated values, systolic BP was not altered by the 1 or 2 mg/kg/day dose of nicotine over the 2-week duration of the study (Fig. 1A). Except for a significant increase in HR caused by the 2 mg/kg/day dose of nicotine after 10 days, HR of female rats treated chronically with nicotine was not statistically different from that of saline-treated rats (Fig. 1B). Plasma cotinine measured 14 days after nicotine administration (0.5, 1 or 2 mg/kg/day) amounted to 75±27, 163±63 and 217±78 ng/ml, respectively.

Effect of chronic nicotine on baroreflex responsiveness

Figures 2-5 depict the effects of nicotine on peripherally-mediated pressor (PE) and depressor (SNP) responses and associated baroreflex-mediated chronotropic responses before and after autonomic blockade. The i.v. administration of PE or SNP (1-16 μg/kg each) elicited dose-dependent pressor (Fig. 2A) and depressor (Fig. 2B) responses, respectively, which were associated with reciprocal changes in HR (Fig. 2C, 2D). The pressor effects of PE were not altered by nicotine, except for a significant decrease in the response to the 16 μg/kg dose of PE in rats receiving the middle dose of nicotine (Fig 2A). Further, the depressor actions generated by the 8 and 16 μg/kg doses of SNP were increased by highest dose of nicotine (Fig. 2B).

On the other hand, reflex bradycardic (PE, Fig. 2C) and tachycardic (SNP, Fig. 2D) responses were significantly reduced in nicotine-treated rats. This resulted in upward (PE) and downward (SNP) shifts in baroreflex curves (Fig. 3A), and dose-related reductions in the slopes of the curves (regression coefficient), which represented the BRS (Fig. 3B). Similar upward and downward shifts in baroreflex curves of PE and SNP, respectively (Fig. 4), and reductions in BRS (Fig. 5) were seen when control rats were treated with atropine or propranolol, suggesting
the importance of cardiac autonomic reflexes (vagal and sympathetic) in these responses. Simultaneous treatment with atropine and propranolol significantly reduced reflex HR responses ($BRS_{PE}$ from $-2.5\pm0.3$ to $-0.4\pm0.1$ mmHg/beats/min, $BRS_{SNP}$ from $-2.8\pm0.1$ to $-0.5\pm0.1$ mmHg/beats/min). This represented approximately 85% reduction in baroreflex gain. In nicotine (2 mg/kg/day for 14 days)-treated rats, reflex HR responses and slopes of the regression lines (BRS) were significantly reduced in presence of propranolol (Figs. 4B,5) but not atropine (Figs. 4A,5). The treatment with atropine or propranolol caused increases and decreases, respectively, in basal HR that were of similar magnitudes in sham rats treated with or without nicotine (Table 1). On the other hand, autonomic blockade by atropine or propranolol elicited minimal changes in basal MAP (Table 1).

**Estrogenic modulation of the autonomic and baroreflex effects of nicotine**

The effects of OVX and treatments with 17β-estradiol or the estrogen receptor modulator raloxifene on BRS in the absence and presence of atropine are illustrated in figure 6. OVX significantly reduced $BRS_{PE}$ (Fig. 6A) but not $BRS_{SNP}$ (Fig. 6B). The OVX-evoked reductions in $BRS_{PE}$ were abolished after 5-day treatment with 17β-estradiol (50 μg/kg/day) or raloxifene (10 mg/kg/day) (Fig. 6A). Further, while nicotine (2 mg/kg/day for 14 days) failed to alter reflex chronotropic responses in OVX rats, it significantly reduced both $BRS_{PE}$ (Fig. 6A) and $BRS_{SNP}$ (Fig. 6B) in 17β-estradiol- or raloxifene-treated OVX rats. In the presence of atropine, similar $BRS_{PE}$ and $BRS_{SNP}$ values were demonstrated in all rat preparations including sham-operated and OVX rats treated with or without nicotine and/or 17β-estradiol (Fig. 6). The increases caused by atropine in basal HR were similarly seen in OVX and OVXE2 rats (i.e. estrogen-independent) and were comparable to those observed in intact sham-operated rats (Table 1). Chronic nicotine significantly attenuated reflex bradycardic and tachycardic responses in proestrus rats in contrast to no effect in diestrous rats (Fig. 7).
Discussion

The current study reports on the dose, hormonal, and autonomic dependences of the interaction of chronic nicotine with reflex HR control in female rats. Chronic nicotine dose-dependently attenuated reflex HR responses in sham-operated rats. The inhibition of the estrogen-mediated vagal facilitation constitutes the underlying mechanism of the baroreflex action of nicotine because: (i) blockade of β-adrenergic, but not muscarinic, receptors caused more deterioration of baroreflex responsiveness in nicotine-treated rats, which highlights the impairment of vagal activity by prior exposure to nicotine, (ii) baroreflex impairment by nicotine disappeared in OVX rats and restored upon treatment with estrogen or raloxifene, and (iii) similar baroreflex gain was demonstrated in all atropine-treated rats regardless of the nicotine or estrogen status.

The dose and duration of the smoking or nicotine regimen greatly impact the nature and magnitude of the evoked cardiovascular abnormalities (Czernin and Waldherr, 2003; Hanna, 2006). In agreement with this, findings of the current and previous (El-Mas et al., 2011c) studies established quiet distinct effects for acute and chronic nicotine on reflex chronotropic responses and autonomic and estrogenic modulation of these responses. For instance, in contrast to no effect for acute nicotine on baroreflexes in female rats (El-Mas et al., 2011c), the current study demonstrated that chronic nicotine dose-dependently impaired reflex bradycardic and tachycardic responses. Another important difference resided in the way by which the estrogen status modulated the baroreflex depressant effects of acute and chronic nicotine. Estrogen protected against the acute nicotine-evoked baroreflex impairment because the latter was observed in OVX rats but not in SO or OVXE2 rats (El-Mas et al., 2011c). This contrasts with the current observation that chronic nicotine reduced BRS in estrogen-repleted (OVXE2 and proestrus) but not -depleted rats (OVX and diestrus), thereby inferring the importance of
estrogen in uncovering the nicotine-induced baroreflex dysfunction. Together, these findings suggest contrasting modulatory effects for estrogen on the baroreflex depressant of acute (protection) and chronic (exacerbation) nicotine.

Reflex HR responses to abrupt perturbations in BP are believed to involve opposite changes in cardiac vagal and sympathetic activities (El-Mas et al., 2001, 2002a; Michelini, 2007). This is supported by the current observations that reflex changes in HR were (i) remarkably reduced after muscarinic (atropine) or β-adrenergic (propranolol) blockade, and (ii) virtually abolished after total autonomic blockade. Notably, the residual HR activity demonstrated in rats treated simultaneously with atropine and propranolol may suggest the incomplete obliteration of autonomic activity or possibly reflects the involvement of direct cardiac effects in chronotropic response to phenylephrine (Posner et al., 1984) or nitroprusside (Herring et al., 2001).

Comparisons of current and previous studies (El-Mas et al., 2011c) also revealed strikingly different contributions of the two divisions of the autonomic nervous system to baroreflex dysfunction caused by acute and chronic regimens of nicotine. In our previous studies (El-Mas et al., 2011c), pharmacologic interruption of reflex cardiac sympathetic, and not vagal, activity mediates the depressant effect of acute nicotine on BRS. Conversely, the current observation that the baroreflex depressant effect of nicotine was abolished in atropine-treated SO rats highlights a pivotal role for the inhibition of the estrogen-dependent cardiac vagal activity in baroreflex dysfunction caused by chronic nicotine. Indeed, our data showed that the BRS depressant effect of atropine in animals treated with nicotine or saline was similar (~ 65% reduction). It is reasonable, therefore, that nicotine produced a maximal inhibition of reflex vagal activity in such way that the concomitant use of atropine could not depress it further. By the same token, the preservation of baroreflex attenuating capacity of nicotine in propranolol-treated
rats rules out a potential role for reflex sympathetic activity in the nicotine-baroreflex interaction. It is conceivable, therefore, to suggest that the presence of functional cardiomotor vagal activity is mandatory for the elicitation of the baroreflex depressant action of chronic nicotine. Notably, because the changes in basal HR caused by atropine (increases) or propranolol (decreases) were similarly demonstrated in sham rats treated with or without nicotine, they are unlikely to contribute to depressant effect of nicotine on baroreflexes. Consistent with our previous report (El-Mas et al., 2011a), these data infer a role for central rather than peripheral (e.g. abundance of cardiac muscarinic or β1-adrenergic receptors) mechanisms in the nicotine-baroreflex interaction. Alternatively, the data also indicate that unlike its depressant effect on rapid adjustments in baroreceptor tone, nicotine does not appear to modify the tonic autonomic control of HR.

The current findings that reflex bradycardia was preferentially impaired in OVX rats and restored to SO levels after estrogen replacement is coherent with a tonic facilitatory effect of estrogen on baroreceptor-mediated HR control. Similar observations were reported in previous studies in which baroreflex function was assessed by the vasoactive (Mohamed et al., 1999; Saleh et al., 2000) or spectral methods (El-Mas and Abdel-Rahman, 2009). The demonstration by Pamidimukkala et al. (2005) that estrogen replacement enhances baroreflex gain in the wild-type OVX rats but not in the estrogen receptor-α knockout OVX rats directly implicates estrogen receptor-α in the baroreflex response to estrogen. Additionally, we report here that the estrogen status of female rats modulate the vagally-dependent baroreflex depressant effect of nicotine. This is convincingly supported by the observations that the atropine-sensitive baroreflex depressant effect of nicotine disappeared in OVX rats and reappeared after replacement of these rats with estrogen or the estrogen receptor modulator raloxifene. In fact, the findings that comparable BRS values were seen in all atropine-treated preparations (SO, OVX, and OVXE2
with or without nicotine; see figure 6) indicate that differences in baroreflex responsiveness among these preparations relate, at least partly, to discrepancies in vagal activity.

Recent anatomical and functional evidence implicates homomeric and heteromeric nAChRs in central autonomic homeostasis. For example, α7 and β2 nAChRs are identified in the nucleus of the solitary tract and dorsal motor nucleus of the vagus, medullary nuclei that are fundamentally involved in the processing of visceral sensory information (Browne et al., 2010; Anfinogenova et al., 2011). Moreover, the area postrema is endowed with the heteromeric α3β4 nAChRs, which modulates the inhibitory GABAergic transmission (Kawa et al., 2007). The following observations demonstrate that the interaction of nicotine with nAChRs varies depending on the receptor, gender, and hormonal profiles: (i) the medullary expression of α7 and β2 nAChRs is decreased and increased, respectively, by nicotine (Browne et al., 2010), (ii) nicotine increases α4β2 nAChR binding sites in mouse brain (Marks et al., 2011), (iii) compared to males, nicotine-treated females have higher medullary β2 nAChRs (Browne et al., 2010), and (iv) estradiol increases α7 nAChRs in dorsal raphe and locus coeruleus neurons (Centeno et al., 2006). More studies are obviously needed to determine the relative contributions of central nicotinic receptors in the gender and hormonally-dependent nicotine-baroreflex interaction.

The arterial baroreflex system is a powerful homeostatic mechanism that negatively correlates with BP. Whereas persistent falls in BP develop upon long-term baroreceptor activation (Lohmeier and Iliescu, 2011), impairment of baroreceptor function is usually associated with hypertension in human (Bristow et al., 1969; Goldstein, 1983) and animal (Gordon et al., 1981; Gordon and Mark, 1983) studies. In the current study, despite the noticeable impairment of baroreceptor function in nicotine-treated rats, tail cuff measurements revealed no changes in systolic BP. Because nicotine was administered in single daily doses, it could be argued that the rapid elimination of nicotine might have hampered the development of
hypertension. Remarkably, the single daily dose regimen has been repeatedly used for studying the biological effects of nicotine (Hui and Ogle, 1991; Ferrari and Fior-Chadi, 2007; El-gowilly et al., 2008). Moreover, pharmacokinetic studies showed that despite the relatively short half-life of nicotine (~ 1 hr), the half-life of cotinine, the principal metabolite and pharmacologically active form of nicotine, ranges from 10 to 24 hours (Miller et al., 1977; Buccafusco and Terry, 2003). In the current study, plasma cotinine levels measured 2-3 hr after dosing with nicotine mimic levels achieved in humans after moderate cigarette smoking (Roethig et al., 2009; Morin et al., 2011), which establishes the clinical relevance of the current study. Our findings, nevertheless, should not be interpreted to preclude a possible correlation between BP and baroreflex activity. Indeed, baroreflex dysfunction precedes the development of hypertension (Gordon et al., 1981; Gordon and Mark, 1983; Shaltout et al., 2012). Probably, a longer period of nicotine exposure might be necessary for revealing nicotine hypertension as reported elsewhere (Ferrari and Fior-Chadi, 2007).

Collectively, the current study showed that the baroreflex effects elicited by chronic nicotine in female rats were qualitatively and quantitatively different from those caused by acute nicotine in rats of the same sex and strain. While reflex chronotropic responses were not affected by acute nicotine (El-Mas et al., 2011c), they were remarkably and dose-dependently attenuated by chronic nicotine (this study). Moreover, the data revealed different modulatory roles for estrogen on baroreflex impairment caused by acute (protection; El-Mas et al., 2011c) and chronic nicotine (exacerbation; current study). Finally, the inhibition of cardiomotor vagal activity mediated the estrogen-dependent baroreflex suppression caused by chronic nicotine. The data emphasize the importance of nicotine regimen in defining autonomic and cardiovascular consequences of the drug.
Authorship Contributions

Participated in research design: El-Mas, El-Gowelli, and El-Gowilly.

Conducted experiments: Fouda and Helmy

Contributed new reagents or analytic tools:

Performed data analysis: El-Mas, El-Gowelli, and El-Gowilly.

Wrote or contributed to the writing of the manuscript: El-Mas, El-Gowelli, and El-Gowilly.

Other: El-Mas received the research funding.
References


Footnotes

This work was supported by the Science and Technology Development Fund, Egypt [STDF No. 502].
Legends for figures

**Figure 1.** Tail-cuff measurements of systolic blood pressure and heart rate (HR) in female rats treated with nicotine (0.5, 1 or 2 mg/kg/day) or saline for 14 consecutive days. Values are means ± SEM of 6-8 observations. *P<0.05 vs. saline values.

**Figure 2.** Effect of nicotine (0.5, 1, or 2 mg/kg/day for 14 days) on increases or decreases in mean arterial pressure (MAP) elicited by phenylephrine and sodium nitroprusside (1-16 μg/kg each), respectively, and associated changes in heart rate (HR) in female rats. Values are means ± SEM of 6-8 observations. *P<0.05 vs. saline values.

**Figure 3.** Baroreflex curves (panel A) generated by phenylephrine and sodium nitroprusside in female rats treated with nicotine (0.5, 1, or 2 mg/kg/day) or saline for 14 days. Panel B shows the slopes of baroreflex curves, which represent baroreflex sensitivity (BRS). Values are means ± SEM of 6-8 observations. *P<0.05 vs. saline values.

**Figure 4.** Effect of muscarinic (atropine, panel A) or β-adrenergic (propranolol, panel B) blockade on baroreflex curves generated by phenylephrine and sodium nitroprusside in female rats treated with nicotine (2 mg/kg/day) or saline for 14 days. Values are means ± SEM of 6-8 observations.

**Figure 5.** Effect of muscarinic (atropine, 1 mg/kg, panel A) or β-adrenergic (propranolol, 1 mg/kg, panel B) blockade on the nicotine (2 mg/kg/day for 14 days)-induced attenuation of baroreflex sensitivity (BRS) in female rats. Values are means ± SEM of 6-8 observations. *P<0.05 vs. saline values, *P<0.05 vs. “saline+atropine” or "saline+propranolol" values.

**Figure 6.** Effect of muscarinic blockade with atropine (1 mg/kg) on the nicotine (2 mg/kg/day for 14 days)-induced reductions in reflex bradycardic (BRS<sub>PE</sub>) and tachycardic (BRS<sub>SNP</sub>) responses in female rats. Values are means ± SEM of 6-7 observations. *P<0.05 vs. sham,
$P<0.05$ vs. OVX, $^+P<0.05$ vs. OVXE2, $^\&P<0.05$ vs. respective “OVX+raloxifene” values,

$^\#P<0.05$ vs. respective “before atropine” values.

**Figure 7.** Baroreflex curves (panel A) generated by phenylephrine and sodium nitroprusside in proestrus and diestrus rats treated with nicotine (2 mg/kg/day) or saline for 14 days. Panel B shows the slopes of baroreflex curves, which represent baroreflex sensitivity (BRS). Values are means ± SEM of 6 observations. $^*P<0.05$ vs. values in saline-treated proestrus rats.
Table 1. Changes in mean arterial pressure (mmHg) and heart rate (beats/min) caused by muscarinic (atropine) or β-adrenergic (propranolol) blockade.

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<th>Group</th>
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<td>Mean arterial pressure</td>
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<td>Sham</td>
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<td>Sham/nicotine</td>
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<td>-1±1</td>
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<tr>
<td>OVX</td>
<td>1±2</td>
<td>-2±2</td>
</tr>
<tr>
<td>OVXE2</td>
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<td>-1±1</td>
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<tr>
<td>Heart rate</td>
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<tr>
<td>Sham</td>
<td>20±4</td>
<td>16±2</td>
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<tr>
<td>Sham/nicotine</td>
<td>21±4</td>
<td>12±2</td>
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<tr>
<td>OVX</td>
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<td>OVXE2</td>
<td>26±10</td>
<td>14±6</td>
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</tbody>
</table>

Values are means±SEM of 6-8 observations.
Fig. 1

A. Systolic pressure (mmHg)

- **Saline**
- **Nicotine 0.5 mg/kg/day**
- **Nicotine 1 mg/kg/day**
- **Nicotine 2 mg/kg/day**

B. HR (beats/min)

- **Saline**
- **Nicotine 0.5 mg/kg/day**
- **Nicotine 1 mg/kg/day**
- **Nicotine 2 mg/kg/day**

Time (days)
Fig. 2

A

$\Delta$ MAP (mmHg)

 Phenylephrine ($\mu$g/kg)

B

$\Delta$ HR (beats/min)

 Nitroprusside ($\mu$g/kg)

 C

 D

Saline
Nicotine 0.5 mg/kg
Nicotine 1 mg/kg
Nicotine 2 mg/kg

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Fig. 4

Nitroprusside vs. Phenylephrine

**A**

- **Δ HR**
  - Saline: ○
  - Nicotine: ●
  - Saline+Atropine: △
  - Nicotine+Atropine: ▲

**B**

- **Δ HR**
  - Saline: ○
  - Nicotine: ●
  - Saline+Propranolol: △
  - Nicotine+Propranolol: ▲

**Δ MAP**
Fig. 5

**A**

- $\text{BRS}_{\text{PE}}$

<table>
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<th>Condition</th>
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<th>Value 3</th>
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<tbody>
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<tr>
<td>Nicotine</td>
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<tr>
<td>Saline+Atropine</td>
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<td>Nicotine+Atropine</td>
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</table>

**B**

- $\text{BRS}_{\text{SNP}}$

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<td>Nicotine+Prop</td>
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Fig. 7

A Nitroprusside Phenylephrine

$\Delta HR$

-120 -80 -40 0 40 80

-40 -20 0 20 40 60

- Proestrus, saline
- Proestrus, nicotine
- Diestrus, saline
- Diestrus, nicotine

B $\Delta MAP$

- Proestrus, saline
- Proestrus, nicotine
- Diestrus, saline
- Diestrus, nicotine

- BRS

Nitroprusside Phenylephrine