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Cardiovascular, Neuropeptide Y, and Adrenergic Responses in Stress Are Sexually Differentiated

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ZUKOWSKA-GROJEC, Z., G. H. SHEN, P. A. CAPRARO AND C. A. VAZ. *Cardiovascular, neuropeptide Y, and adrenergic responses in stress are sexually differentiated.* PHYSIOL BEHAV **49**(4) 771–777, 1991.—Cardiovascular and sympatho-adrenomedullary responsiveness at rest and during stress were studied in weight-matched, sexually mature male and female rats. At rest, although there were no sex differences in cardiovascular parameters, females had two-fold higher plasma levels of norepinephrine (NE) and epinephrine. Resting plasma levels of neuropeptide Y-immunoreactivity (NPY-ir, a putative sympathetic cotransmitter and a vasoconstrictor) were similar in both sexes. Stresses of handling and cold (4°C) water exposure induced greater pressor and tachycardic responses in males than in females. Males but not females exhibited a protracted recovery from the stress-induced pressor responses and a 2-fold increase in plasma NPY-ir suggesting that NPY release is sexually differentiated. Only in males, low basal plasma NE and NPY-ir levels inversely correlated with greater cold-induced pressor responses. Furthermore, in areflexic pithed rats, pressor adrenergic and NPY responses were greater in males than in females suggesting the possibility of "down"-regulation of vascular adrenergic receptors in females (due to elevated circulating catecholamines) and "up"-regulation of NPY and adrenergic receptors in males.

Sex Stress Norepinephrine Epinephrine Neuropeptide Y Cardiovascular Sympatho-adrenomedullary Blood pressure Heart rate

STRESS is recognized to play a permissive role in the pathophysiology of cardiovascular diseases, although not as an "official risk factor." Hyperreactivity to stress such as a cold pressor test is predictive of subsequent coronary heart disease (24). In premenopausal women, cardiovascular responses to numerous environmental challenges (7,19), including cold temperature (25), are less intense than those of men, and the prevalence of coronary heart disease is also lower than in men (12). Thus sex may be an important factor influencing both the stress response and cardiovascular pathophysiology. Although rats are the most frequently used animal model for stress studies, little is known about sex differences in cardiovascular responsiveness to stress in these animals.

The sympatho-adrenomedullary system responds to stress with the release of not only catecholamines, norepinephrine (NE) and epinephrine (EPI), but also of neuropeptide Y (NPY). This peptide contains 36 amino acids (23) and is costored and coreleased with NE from the postganglionic sympathetic nerves innervating the cardiovascular system (6), it is present in the adrenal medulla, and, as our recent studies revealed, also in platelets (17). In the sympathetic nerves, NPY is considered a putative cotransmitter, exerting potent direct vasoconstrictor action but also potentiating NE-induced vasoconstriction (30). Reciprocally, elevated catecholamine levels stimulate blood vessels to greater responsiveness to NPY (27). However, the hemodynamic effects of NPY are not identical with NE. NPY causes vasoconstriction in some vessels (e.g., coronary and cerebral) where NE is weakly or not effective (5,20). Furthermore, the peptide has a longer lasting effect, and in some organs its action even opposes that of NE (e.g., cardiodepression in the rat heart) (32).

We (31,33) and others (18) find that NPY is released into the circulation in animals and humans during prolonged and intense stress, and thus may contribute to hemodynamic changes during stress. We have previously shown that different stressors evoke differential plasma catecholamine and NPY responses in male rats (31); this may be due to multiple sources of circulating NPY. Both sympatho-neural activation and platelet aggregation appear to contribute to elevated plasma NPY-ir levels during stress (29).

The purpose of the present study was to determine whether there are sex differences in cardiovascular and plasma NPY-ir re-

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sponses to two types of stress: a mild stress of handling and a transfer to a novel environment (H + N), and a more intense stress of cold (4°C) water exposure. In addition to studying the release of NPY, pressor responses to NPY and NE were determined in male and female rats, in the presence (conscious) and absence (pithed) of circulatory reflexes.

METHOD

Conscious Rat Studies

Weight-matched (16- to 20-week-old), sexually mature male and female Sprague-Dawley rats (Zivic Miller, Allison, PA) were used in the study. Rats were kept in our animal research facility under a 12-h lighting cycle with controlled temperature and humidity, and food and water ad lib for at least 7 days before the experiments.

Twenty-four hours before the experiment the rats were anesthetized with halothane (2% in oxygen) and implanted with an indwelling femoral arterial catheter for blood pressure recording and blood sampling, and a femoral venous catheter for drug administration. The catheters were exteriorized at the back of the neck and protected by spring wires, as described previously (31,33). Following surgery, rats were housed individually in square cages with food and water ad lib, and placed in a quiet room. On the day of the experiment, resting blood pressure (Beckman R611) and heart rate (cardiotachometer) were recorded, and basal blood samples for catecholamines (0.4 ml) and NPY-ir (0.5 ml) were withdrawn while rats were resting in their home cages, screened from the external environment. Here and subsequently, blood was replaced with blood elements from the same animal, resuspended in saline of volume equal to that of the withdrawn plasma. Previous studies have established that this procedure does not affect cardiovascular and catecholamine responses.

Pressor Responsiveness Study in Conscious Rats

Twenty-four to forty-eight hours after the surgery, conscious male and female rats (n = 7 each) were tested for their responsiveness to NE and NPY. Drugs were administered IV in a volume of 0.2 ml of saline, in random order and at increasing doses (0.3–1.0 μg/kg and 0.6–2 nmol/kg, respectively), and at minimum intervals of 30 min to ensure the complete dissipation of the preceding cardiovascular responses. These doses of NE and NPY were chosen based on our previous studies (26,30) as eliciting 30 and 80% of the maximal responses, and being approximately equipotent.

Stress Tests

The stress tests were performed 24–48 hours after the drug administration study. The first stress test consisted of picking up the rats and transferring them from their home cages into a novel environment which was an empty, transparent cage (stress of handling + novelty, H + N). Cardiovascular parameters were recorded continuously whereas blood samples were taken before and at 10 min into the test. Following a complete dissipation of cardiovascular responses (approximately 10–15 min) iced water (4°C) was poured onto the floor of the cage to the depth of 1 cm. Rats were kept in cold water for 10 min and subsequently placed back in their home cages for recovery. Blood samples were withdrawn at the end of cold water test (10 min), and at 10 and 30 min of recovery.

Pithed Rat Study

On the day of the experiments, rats were anesthetized with halothane (2% in oxygen) and the right carotid artery was cannulated for blood pressure recording. Heart rate was computed by the cardiotachometer. Drugs were administered via a jugular vein catheter. Bilateral vagotomy was performed at the midcervical level and the trachea cannulated. Rats were pithed with a steel rod as previously described (26) and anesthetic discontinued. The rats were ventilated with air enriched with oxygen at 1 ml/100 g body weight per stroke at a rate of 55 strokes/min, and rectal temperature was maintained at 37°C by a heating pad. After 30 min of stabilization period, NE and NPY were administered intravenously at random order, at increasing doses of 0.1–1.0 μg/kg and 0.2–2.0 nmol/kg, respectively.

Plasma Catecholamine Assay

Samples (0.4 ml) of arterial blood were withdrawn by free flow via the femoral artery catheter into heparinized tubes while the rats were resting in their home cages. Plasma was separated from cell elements by centrifugation at 4°C and aliquots (120 μl) of plasma were stored in −70°C until the assay. NE and EPI were measured in plasma using reversed phase high pressure liquid chromatography with electrochemical detection after batch alumina extraction, as previously described (4). The limit of detection was about 50 pg/ml for NE and EPI.

Plasma NPY-ir Assay

Plasma NPY-ir was measured by a radioimmunoassay using NPY-specific antiserum, porcine synthetic NPY, and ^{125}I-radiolabelled NPY (Peninsula Lab., Belmont, CA) (31). Arterial blood samples were collected by free flow into ice-chilled tubes containing 0.5 trypsin inhibitory unit of Aprotinin and 2.25 mg EDTA. Plasma was stored in −70°C until the assay, extracted using acid ethanol and dried by Speed-Vac. The limit of detection of NPY-ir in plasma was about 20 fmol/ml.

Statistical Evaluation of Data

Data were analyzed by analysis of variance for repeated measures, followed, where appropriate, by one-way ANOVA and post hoc Dunnett's *t*-test using StatView 512+ with an Macintosh computer. Relationship between plasma catecholamines, NPY-ir and cardiovascular parameters were analyzed by simple regression using StatView 512+. Results were considered significant if $p<0.05$, and are reported as mean ± standard error, for the indicated number of rats.

RESULTS

Cardiovascular Responses to Stress

At rest, there were no significant differences in mean arterial pressure (MAP) or heart rate (HR) between weight-matched male and female rats (females 2–4 week older than males). The stress of handling and transfer to a novel environment increased MAP significantly more in males (from 104.2 ± 2.1 to 125.5 ± 2.2 mmHg) than in females (from 102.7 ± 2.0 to 114.9 ± 2.1 mmHg) (between the groups one-factor ANOVA: $F = 11.519$, post hoc *t*-test: $p<0.05$, Fig. 1). Tachycardic responses to H + N were also greater in males (from 380 ± 8 to 480 ± 12 beats/min) than in females (from 364 ± 13 to 419 ± 10 beats/min); between the groups one factor ANOVA: $F = 13.0$, post hoc *t*-test: $p<0.05$, Fig. 1).

Males responded to the cold water test with greater increments of MAP (by 35.4 ± 1.7 mmHg) than did females (by 27.1 ± 3.0 mmHg, ANOVA: $F = 8.04$, post hoc *t*-test: $p<0.05$, Fig. 1). Furthermore, stress-induced pressor responses lasted longer in males than in females; at 30 min MAP remained elevated in males

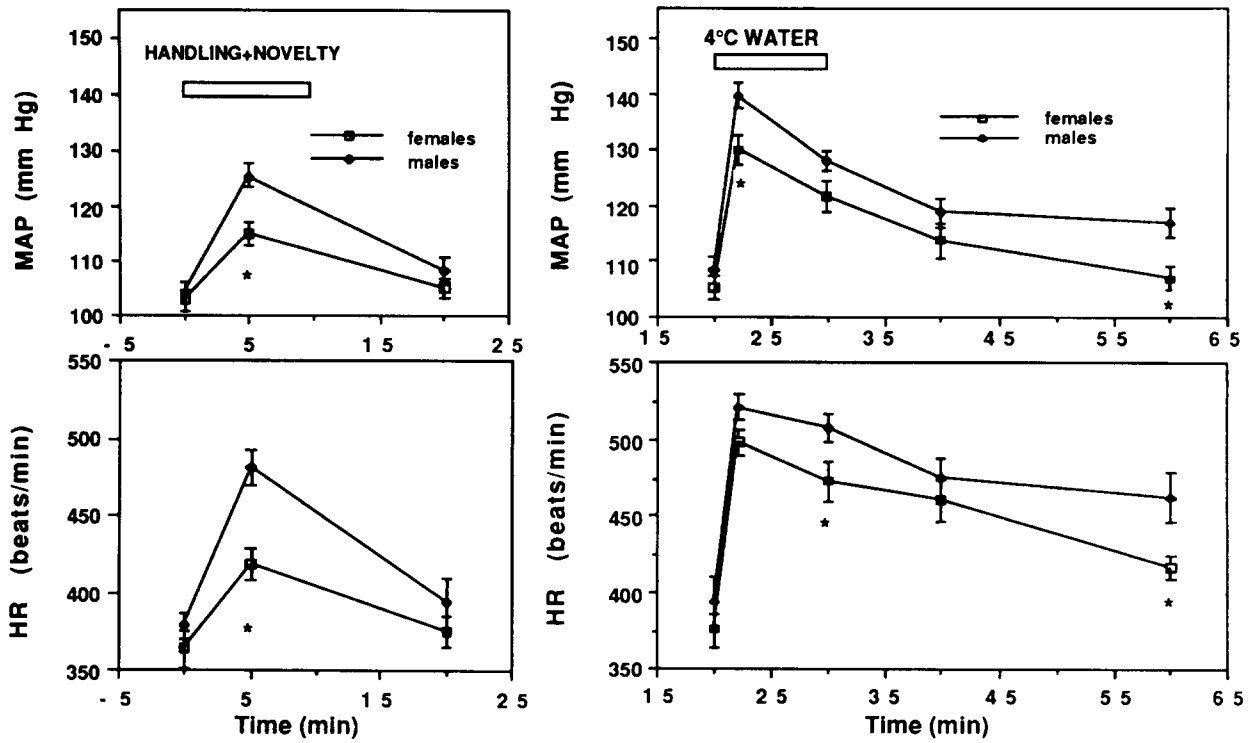

FIG. 1. Cardiovascular responses to stress of handling + novel environment (H + N) and cold (4°C) water test (COLD) in conscious male and female rats (n = 7 each group). Upper panels: mean arterial pressures (MAP); lower panels: heart rates (HR). Changes in MAP were significant by two-factor ANOVA for repeated measures: for group ($F = 6.008$, $p = 0.027$), time ($F = 106.923$, $p = 0.0001$) and group-time interactions ($F = 2.696$, $p = 0.0188$). Changes in HR were significant for group ($F = 8.0$, $p = 0.0111$) and time ($F = 52.0$, $p = 0.0001$), and for group-time interactions ($F = 1.0$, $p = 0.1888$, by ANOVA for repeated measures). Asterisks denote the level of statistical significance of difference between males and females by one-factor ANOVA followed by a post hoc Dunnett's t-test: $*p < 0.05$.

(116.8 ± 2.6 mmHg), whereas in females MAP normalized (106.9 ± 2.1 mmHg). The summated total pressor response (area under the curve relating increments in MAP to time, for a total of 60 min) was 2-fold greater in males than in females. In contrast, although the peak increase in heart rate was greater in males than in females (Fig. 1), the summated tachycardic responses to stress were not different between the sexes.

Plasma Levels of Catecholamines and NPY-ir at Rest

Figure 2 shows resting plasma levels of catecholamines and NPY-ir in both sexes. Females had significantly higher circulating levels of NE (2.41 ± 0.31 versus 1.27 ± 0.16 pmol/ml, $p < 0.02$) and EPI (1.51 ± 0.29 versus 0.65 ± 0.11 pmol/ml, $p < 0.05$). In contrast, there were no differences between plasma NPY-ir levels in males and females (Fig. 2).

Plasma NPY-ir Responses to Stress

In females, plasma NPY-ir levels increased only during the first stress test which was H + N (by 0.40 ± 0.18 pmol/ml, ANOVA for repeated measures: $F = 2.492$, post hoc t-test: $p < 0.05$, Fig. 3) but normalized thereafter. Males did not respond to H + N with any increase in plasma NPY-ir. However, in males, circulating NPY-ir levels continued to rise during cold water stress, and the peak increase occurred at 30 min of recovery reaching significantly higher levels than in females ($p < 0.05$, Fig. 3).

Relationship Between Resting Plasma Levels of NE, EPI, NPY-ir and Pressor Responsiveness to Stress

In females, there was no correlation between resting plasma NE levels and the stress (cold)-induced pressor responses [$r = .431$, $F(5) = 0.913$, $p = 0.393$, Fig. 4]. In contrast, in males, basal plasma NE levels inversely correlated with the increases in MAP induced by cold stress [$r = -.871$, $F(5) = 12.623$, $p = 0.023$,

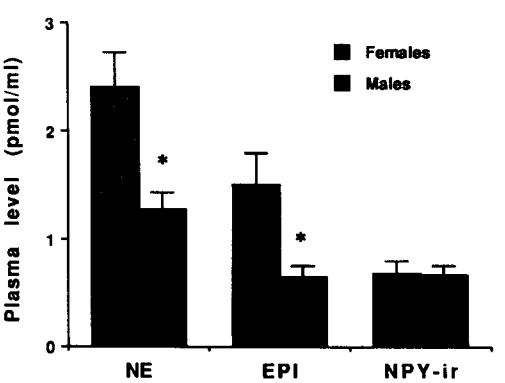

FIG. 2. Resting circulating plasma levels of catecholamines and NPY-ir in conscious male and female rats. Asterisks denote the level of statistical significance of differences between male and female rats (n = 15 each): $*p < 0.05$ by Student's t-test.

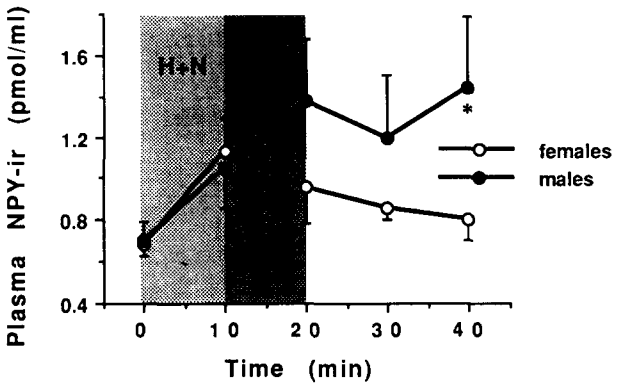

FIG. 3. Plasma NPY-ir responses to stress of handling + novel environment (H + N) and cold (4°C) water exposure (COLD) in male and female rats (n = 7 each group). Changes in plasma NPY-ir levels were significant by two-factor ANOVA for repeated measures for time ($F = 2.992$, $p = 0.049$), while for group ($F = 4.1$, $p = 0.0624$), and for group-time interaction ($F = 1.419$, $p = 0.2395$). Asterisk denotes significant difference by one-factor ANOVA and post hoc Dunnett's t-test between males and females: $*p < 0.05$.

Fig. 4]. A similar relationship was found in regard to resting plasma NPY-ir levels and pressor responsiveness (Fig. 5). In males, basal NPY-ir levels inversely correlated with the stress (cold)-induced pressor responses [$r = -.848$, $F(7) = 15.332$, $p = 0.007$, Fig. 5], whereas no statistically significant relationship was found in the females [$r = .386$, $F(6) = 0.877$, $p = 0.392$, Fig. 5]. No correlation was found between resting plasma levels of NE and NPY-ir due to the small number of rats where both parameters were available. Furthermore, there was no correlation between the cold stress-induced increases in plasma NPY-ir levels and pressor responses to stress (males: $r = .176$, females: $r = .201$).

No statistically significant relationship was found between resting plasma EPI levels and pressor responsiveness to cold stress (males: $r = .41$, $p = 0.492$, females: $r = .084$, $p = 0.857$).

Pressor Responsiveness to NE and NPY in Conscious and Pithed Rats

In conscious rats, pressor responses to NE were not statistically different between males and females (at 1.0 μg/kg: 62.9 ± 4.1 and 58.8 ± 3.4 mmHg, respectively, Fig. 6). Similarly, pressor responses to NPY did not differ between conscious male and female rats (Fig. 7). In contrast, in pithed male rats, NE administration (0.1–1.0 μg/kg) evoked dose-dependent pressor responses which were significantly greater in male than in female rats (83.8 ± 8.5 and 66.3 ± 8.4 mmHg, respectively, by one-factor ANOVA: $F = 7.945$; post hoc t-test: $p < 0.05$, at the highest dose of NE, Fig. 6). Likewise, the NPY (0.2–2.0 nmol/kg) dose-response curve was shifted to the left in males compared to pithed females (63.0 ± 6.2 and 38.7 ± 6.9 mmHg, by ANOVA: $F = 7.741$; and t-test: $p < 0.05$, at the highest dose of NPY, Fig. 7).

DISCUSSION

The results of our study indicate that males have augmented and more prolonged cardiovascular responses to acute stress when compared with females. The study was strengthened in that sex differences were found using two different stressors, handling of rats with exposure to a novel environment, and the cold water test. Stress also evoked differential plasma NPY-ir responses in males and females.

At rest, MAP and HR of sexually mature, weight-matched male and female rats were similar. However, circulating levels of catecholamines were unexpectedly found to be 2-fold higher in females than in males. The marked elevation of plasma EPI suggests greater adrenomedullary activity in the females than in the males. Increased resting levels of circulating NE could also result from adreno-medullary hyperreactivity, or greater sympatho-neural activity in females.

A measure of sympatho-neural activity could be derived from changes in plasma NPY-ir levels. Previously, our (31) and other (18) studies showed that increases in plasma NPY-ir correlate with those of NE but not with EPI during stress in rats and humans. Moreover, removal of the adrenal medulla in rats was found to be ineffective in lowering basal plasma NPY levels (10) and enhanced, instead of inhibiting, stress-induced plasma NPY responses (16). These findings support the notion that NPY is released from the sympathetic nerves (14) and not from the adrenal medulla. Accordingly, in our study, similar resting plasma NPY-ir levels in both sexes may indicate similar sympathetic nerve activity. If so, increased plasma NE levels in females would rather imply decreased NE inactivation (decreased uptake-1 and -2), not increased release. Conversely, it could indicate that increased release of NPY in females is offset by increased inactivation of the peptide. However, little is known about the latter.

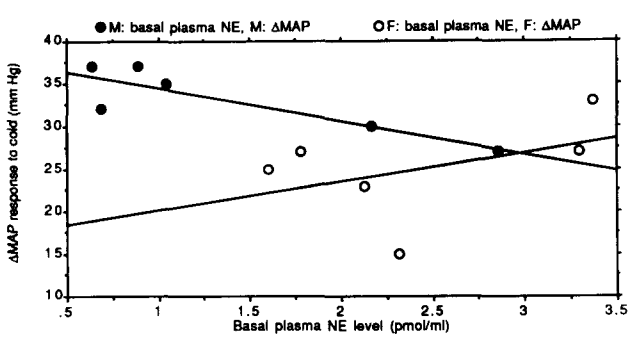

FIG. 4. Correlation between basal plasma NE levels and increases in mean arterial pressure (MAP) induced by cold stress in males (M, filled circles) and females (F, open circles). Males: $y = -3.853x + 38.324$, $r = .871$; females; $y = 3.386x + 16.817$, $r = .431$.

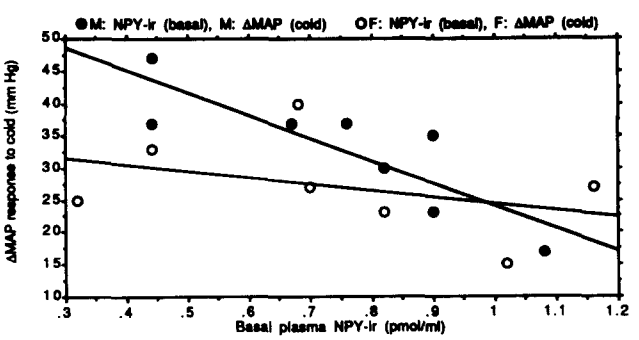

FIG. 5. Correlation between basal plasma NPY-ir levels and increases in MAP induced by cold stress. Males (M): $y = -35.103x + 59.246$, $r = -.848$; females (F); $y = -10.166x + 34.608$, $r = .386$.

Sex differences in the inactivation of catecholamines are likely since estrogens are potent (more so than testosterone) inhibitors of the extraneuronal uptake of NE (uptake-2), and additionally, they weakly inhibit intraneuronal NE uptake (uptake-1) (22). Moreover, there is some evidence (in urogenital organs) that estrogens inhibit, not increase, the neuronal release of NE (2). Conversely, estrogens increase the adreno-medullary secretion of catecholamines (28). This would support our conclusion that, at rest, females have higher circulating catecholamine levels due to greater adreno-medullary activity as compared to males but have similar sympathoneural activity.

Pressor and tachycardic responses to both stressors, handling + novelty and cold water exposure, were significantly greater in males than in females. In addition, the summated pressor response, over a period of 60 min, was 50% greater in male than in female rats. Since the summated tachycardic responses were not different between the sexes, this suggests that one component of the male-female difference in hypertensive responses to stress is not dependent on tachycardia but on changes in vascular resistance. Even if the sex differential in maximal cardiovascular responses were subtle, the analysis of the duration of (total) stress responses reveals marked differences. This lingering poststress increase in MAP in males may have a greater "wear and tear" effect on their cardiovascular system.

Associated with this protracted stress-induced pressor response, there was an increase in plasma NPY-ir in males but not in females. Testosterone increases NPY concentration and release from the hypothalamus of male rats (21) which supports the possibility that synthesis and/or release of NPY may be differentially regulated by sex hormones. Thus NPY may contribute to pressor responses to stress in males considering vasoconstrictor action of NPY, especially since the peptide causes more prolonged vasoconstriction than NE in such vascular beds as the mesenteric and the renal (31). This possibility will await testing until a specific NPY antagonist becomes available.

The maximal increase in plasma NPY-ir levels in males evoked by the cold water stress was delayed in relation to the peak increases in cardiovascular parameters. Although we (31) and others (16) show that plasma NPY responses correlate with sympathetic activation, other possible sources of NPY, including the platelets, should be considered. Our recent finding of the presence of NPY in rat platelets and its release from platelets during aggregation both in vitro (17) and in vivo (29) raises an interesting possibility that circulating NPY-ir levels are, in part, platelet derived, and may contribute to sex differences. Platelet aggregability increases during stress (26). Moreover, platelets from male rats are more aggregable than those of females (1). It remains to be determined whether male platelets release more NPY.

In males, low basal plasma NE and NPY-ir levels correlated with high pressor responsiveness to cold stress. In females, with their high circulating catecholamine levels, plasma NE was not predictive of pressor responsiveness to stress (which was lower than in males). In some studies (13) in which men and women were analyzed together, a positive correlation was shown between resting plasma NE and stress-induced pressor responses. However, it is also known that increased circulating levels of NE, e.g., by infusion of NE or by stress, result in desensitization of alpha-adrenoceptors and lead to attenuated adrenergic responses (11). Thus lower circulating NE levels in males may enhance their vascular alpha-adrenergic reactivity, whereas in females, the reverse may occur. This hypothesis derives support from our vasopressor reactivity studies.

Vascular reactivity to sympathetic activation was found to be greater in kidneys of male compared to female normotensive and hypertensive rats (3). We have also found that pressor responses to adrenergic stimulation in vitro, in the isolated perfused mesenteric bed, are greater in males than in females (submitted for publication). Presently, we show that vasopressor responses to administered NE are augmented in areflexic males compared to the females. These findings and the higher resting levels of circulating catecholamines in females than in males suggest that diminished female adrenergic responsiveness may be due to "down"-regulation of adrenergic receptors. Lower female adrenergic vascular responsiveness is reported in humans (8). Whether this is due to a lower affinity/density of alpha-adrenergic receptors (15) or to a greater release of secondary vasodilator mediators (9) induced by female sex hormones needs to be investigated.

Like NE, pressor responsiveness to NPY administration is enhanced in the males in the absence of circulatory reflexes (pithed) but not in the conscious state. The disappearance of these sex differences in the conscious state may indicate that circulatory reflexes also differ between male and female rats. Additionally, in females, elevated resting circulating catecholamines in the conscious rats (but not in the pithed rats, data not shown) probably enhanced their intrinsically low vascular reactivity to NPY. We find that the exposure of blood vessels to high concentrations of NE markedly enhances responsiveness to NPY, both in vivo and in vitro (10). In contrast, the male responsiveness to NPY was similar in the pithed and the conscious state, and, therefore, may

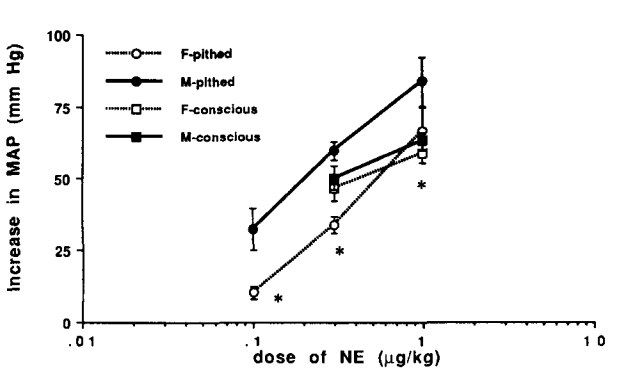

FIG. 6. Pressor responses to NE administration in conscious (n = 7) and pithed (n = 6) male and female rats. Asterisk denotes statistical significance of difference between the sexes by ANOVA (F = 7.945) and Dunnett's *t*-test, $*p<0.05$.

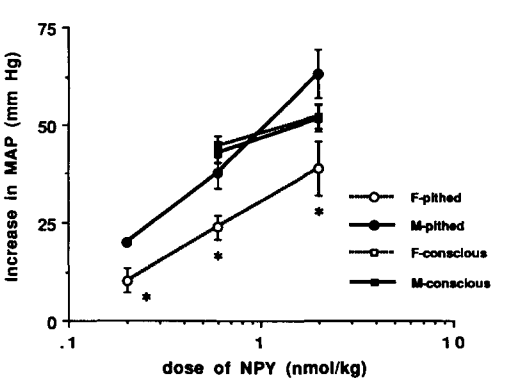

FIG. 7. Pressor responses to NPY administration in conscious (n = 7) and pithed (n = 6) male and female rats. Asterisk denotes statistical significance of difference between the sexes by ANOVA (F = 7.741) and Dunnett's *t*-test, $*p<0.05$.

represent an intrinsic vascular hypersensitivity to NPY. Our findings suggest (but do not prove) the possibility of "up"-regulation of vascular NPY receptors in male rats.

In summary, sexually mature female rats exhibit higher circulating plasma catecholamine levels, probably due to increased adrenomedullary secretion, compared to males. In contrast, males respond to stress with greater increments of plasma NPY-ir levels which may be of sympatho-neural and/or platelet-derived origin, and with greater vasopressor and tachycardic responses than do females. Our findings suggest that this vasoconstrictor peptide may be, in part, responsible for the protracted pressor response in males, given the prolonged vasoconstrictor actions of NPY. The lower cardiovascular responsiveness to stress and the lower vasopressor adrenergic responsiveness in females may be due to "down"-regulation of their adrenoceptors as a result of higher resting circulating catecholamines. Conversely, the greater pressor responsiveness to NPY and NE in males may indicate "up"-regulation of vascular NPY and adrenergic receptors.

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