Delayed stress-induced colonic hypersensitivity in male Wistar rats: role of neurokinin-1 and corticotropin-releasing factor-1 receptors

Ines Schwetz, Sylvie Bradesi, James A. McRoberts, Marciano Sablad, Jerry C. Miller, Huping Zhou, Gordon Ohning, and Emeran A. Mayer. Delayed stress-induced colonic hypersensitivity in male Wistar rats: role of neurokinin-1 and corticotropin-releasing factor-1 receptors. Am J Physiol Gastrointest Liver Physiol 286: G683–G691, 2004. First published November 13, 2003; 10.1152/ajpgi.00358.2003.—The mechanism(s) underlying stress-induced colonic hypersensitivity (SICH) are incompletely understood. Our aims were to assess the acute and delayed (24 h) effect of water avoidance (WA) stress on visceral nociception in awake male Wistar rats and to evaluate the role of two stress-related modulation systems: the substance P/neurokinin-1 receptor (SP/NK1 R) and the corticotropin-releasing factor (CRF)/CRF1 receptor (CRF/CRF1 R) systems, as well as the possible involvement of the sympathetic nervous system. Visceral pain responses were measured as the visceral motor response to colorectal distension (CRD) at baseline, immediately after WA and again 24 h later. The NK1 R antagonists RP-67580 and SR-140333 and the CRF1 R antagonist CP-154526 were injected 15 min before WA or 1 h before the CRD on day 2. Both NK1 R antagonists only decreased SICH when applied before WA stress or before the CRD on day 2. Neither CRF1 R antagonist abolished delayed SICH when applied either before WA stress or before the CRD on day 2. Both NK1 R antagonists increased perception thresholds and increased intensity ratings in response to rectal distension in healthy volunteers (25). Bradesi et al. (2) recently demonstrated that an NK1 R antagonist (SR-140333) significantly counteracted acute restraint stress-induced increases in abdominal contractions to rectal distension (15), and preliminary results suggest that CRF1 R antagonists may modulate pain responses centrally (24) or peripherally (40). For example, central CRF release is associated with activation of the sympathetic nervous system (SNS), as well as with activation of the hypothalamic-pituitary-adrenal axis (6, 29), both of which can have profound effects on peripheral targets. In rats, central administration of CRF mimics the effect of restraint stress in increasing the number of abdominal contractions to rectal distension (15), and preliminary results suggest that CRF1 R antagonists may modulate pain responses centrally (24) or peripherally (40).

CRF is a major mediator of the effects of stress on the gastrointestinal tract involving both central and peripheral CRF receptors (44), and activation of the CRF/CRF1 R system may modulate pain responses centrally (24) or peripherally (40). For example, central CRF release is associated with activation of the sympathetic nervous system (SNS), as well as with activation of the hypothalamic-pituitary-adrenal axis (6, 29), both of which can have profound effects on peripheral targets. In rats, central administration of CRF mimics the effect of restraint stress in increasing the number of abdominal contractions to rectal distension (15), and preliminary results suggest that CRF1 R antagonists may modulate pain responses centrally (24) or peripherally (40).

Address for reprint requests and other correspondence: E. A. Mayer, Center for Neurovisceral Sciences and Women’s Health, Veterans Affairs Greater Los Angeles Healthcare System, Bldg. 115/Center for Ulcer Research and Education, Rm. 223, 11301 Wilshire Blvd., Los Angeles, CA 90073 (E-mail: emayer@ucla.edu).

http://www.ajpgi.org

G683
postganglionic sympathetic nerves could trigger the release of mediators from mast cells, such as tryptase and histamine, or other immune cells that can produce primary afferent sensitization (4). Such SNS-mediated mast cell degranulation has been demonstrated in other organ systems (22).

In the present study, we examined the general hypothesis that activation of NK1Rs and CRF1Rs are involved in the stress-induced increase of colonic nociception in male Wistar rats, a rat strain with increased anxiety-like behavior (14). Viscero-motor response (VMR) to colorectal distension (CRD) was used to assess colonic nociception and water avoidance (WA), an established mild psychological stressor, to address the following questions. First, does an acute psychological stressor produce a delayed increase in colonic nociception, as assessed at 24 h after the stressor? Second, is delayed SICH dependent on NK1Rs and CRF1Rs? Third, do the delayed stress-induced changes in colonic nociception involve peripheral mechanisms such as activation of the SNS? Some of the data presented in this paper have previously been published in abstract form (41).

MATERIALS AND METHODS

Animals

Experiments were performed by using male Wistar rats purchased from Harlan Sprague-Dawley (San Diego, CA; weight, 240–260 g at time of purchase). The rats were housed in pairs with free access to food and water. A 12:12-h light-dark cycle (lights on at 0600 h) was maintained. The animals were allowed to acclimate to the animal facility for 1 wk before surgery. All protocols were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Greater Los Angeles Healthcare System (Los Angeles, CA).

Surgery

As previously described (7), electrodes (Teflon-coated stainless steel; AstraZeneca R&D, Mölndal, Sweden) for electromyographic (EMG) recording were stitched into the external oblique musculature immediately superior to the inguinal ligament. The cannula housing the electrode leads was externalized through a 4-mm incision on the left side of the abdominal wall for access during subsequent experimental measurements. After surgery, rats were housed in pairs and allowed to recuperate for at least 7 days. Wounds were examined to ensure complete recovery before testing.

Assessment of Visceral Sensitivity: CRD

The visceral stimulus employed was distension of the descending colon and rectum by using a method that has been previously described (36). Briefly, animals were lightly anesthetized with halothane and a lubricated flexible latex balloon (6 cm) was inserted intra-anally into the descending colon. The balloon was positioned such that its end was 1 cm proximal to the anus and was secured in place by taping the balloon catheter to the base of the tail. Animals were placed in a Plexiglas cage and allowed to recover for ~30 min. The balloon pressure was continuously monitored online with the aid of a customized pressure control device (AstraZeneca). CRD in awake rats results in contraction of the abdominal and hind limb musculature (36), recorded as EMG activity in the external oblique musculature. Each distension lasted 20 s at 4-min interstimulus intervals. EMG activity was recorded 20 s before CRD, 20 s during CRD, and 20 s after termination of CRD. The EMG activity was rectified, and the increase in the area under the curve (AUC) of EMG amplitude (over baseline) was recorded.

WA Stress

The rats were placed on a pedestal (8 × 8 × 10 cm) attached to the bottom of a plastic tank (25 × 25 × 45 cm). The tank was filled with fresh tap water at room temperature (21°C) within 1 cm of the top of the block. This well-characterized test represents a psychological stressor associated with large elevations of ACTH and corticosterone within 30 min (34). Sham WA, the control condition, consisted of placing rats in the same, albeit dry, plastic tank.

Experimental Protocol

All animals were handled and habituated to the Plexiglas cages for 30 min on three consecutive days including the day before baseline testing.

Effect of stress on VMR. In the first series of experiments, two groups of male Wistar rats (n = 12 in each group) were used. Baseline VMR to graded intensities of phasic CRD (2 × 60 mmHg and two ascending series of 10, 20, 40, and 60 mmHg) were obtained. The same CRD protocol was used throughout the study. One group was submitted to WA stress. The second group was used as a control and subjected to sham WA. The VMR to CRD was recorded immediately after the stress or sham stress procedure and again 24 h later.

Effect of the selective CRF1R antagonist, CP-154526, The first set of experiments consisted of four groups of animals. The first group (n = 12) was injected subcutaneously with the CRF1R antagonist, CP-154526, at 32 mg/kg (dissolved in 10% DMSO, 10% CremophorEL, and saline) 45 min before WA. A second group (n = 11), treated with vehicle, served as controls. The third group (n = 12) was injected with the compound 1 h before the start of the CRD on day 2, with the fourth group (n = 10) as vehicle controls. All experiments were repeated in the sham stress condition to exclude a per se effect of the compound.

Effect of the NK1R antagonists, RP-67580, and SR-140333. The first group (n = 18) received the NK1R antagonist RP-67580 (dissolved in distilled water) at 3 mg/kg or vehicle intraperitoneally 15 min before the WA stress. Ten rats were injected with RP-67580 (3 mg/kg) 1 h before the start of the CRD on the second day. On the basis of the results of the 3-mg/kg dose, another group of 10 animals was injected with 1 mg/kg before the CRD on the second day. Ten rats served as vehicle controls. To exclude a per se effect of the compound, sham stressed animals were injected with 3 mg/kg RP-67580 or vehicle before the 24 h CRD.

To confirm our results with RP-67580 and to rule out possible nonspecific effects of this compound, we repeated the experiments outlined above with another specific NK1R antagonist, SR-140333. Briefly, SR-140333 (1 mg/kg ip, dissolved in saline) or vehicle was injected 15 min before WA (n = 10 in each group). Ten rats were treated with the compound (1 mg/kg) 1 h before CRD on day 2. On the basis of the results of the 1-mg dose, one group of animals was injected with 0.1 mg/kg SR-140333 before the CRD on day 2 (n = 10). A separate group (n = 10) was injected with vehicle. To exclude a per se effect of the compound, sham stressed animals were injected with 1 mg/kg SR-140333 or vehicle before the CRD on day 2.

Chemical sympathectomy. Chemical sympathectomy was performed and validated as previously described (32). Ten rats were injected intraperitoneally with four doses of 6-hydroxydopamine (6-OHDA) dissolved in 0.1% ascorbic acid in saline over 7 days [days 1 and 2, 50 mg/kg; days 6 and 7, 100 mg/kg; total dose 300 mg/kg; protocol modified from McCafferty et al. (32)]. A second group was treated with vehicle. The day after the last injection, a baseline CRD was performed. All animals were submitted to WA. CRD was repeated immediately after WA and again 24 h later. We assessed the effectiveness of chemical sympathectomy by examination of neu- ropeptide Y (NPY) immunoreactivity in perivascular nerves in the submucosa of the colon as previously described (12). Briefly, colonic tissue was pinned flat in a Sylgard petri dish and fixed overnight in 4% paraformaldehyde, 0.1 M PBS, pH 7.4, at 4°C. Fixed tissue was...
subsequently immersed in 20% sucrose at 4°C, embedded with optimal cutting temperature compound embedding medium (Miles, Elkhart, IN), frozen on dry ice, and sectioned with a cryostat at 8 μm thickness.

Immunostaining for NPY was performed by incubating frozen tissue sections overnight at 4°C with rabbit polyclonal NPY antibody [1:1,000; Center for Ulcer Research and Education (CURE) No. 8711] followed by incubation with Rhodamine (tetramethylrhodamine isothiocyanate)-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA) overnight at 4°C. Nonspecific labeling was determined by using primary NPY antibody that had been preincubated with excess NPY peptide for 2 h at room temperature before application to the tissue sections. All tissue sections were washed three times for 10 min in PBS before the addition of subsequent antibodies. Stained sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sections were examined with the examiner blinded to the treatment and analyzed for the degree and intensity of NPY immunostaining.

Drugs

6-OHDA hydrobromide was bought from Regis. The CRF1 antagonist, CP-154526, was a gift from Pfizer. SR-140333 was kindly supplied by SanoSynthalabo France. The NK1R antagonist RP-67580 was a gift from Aventis Pharma. In all experiments, intraperitoneal injections of drug or vehicle were given in a volume of 1 mg/ml. Doses used for each compound were selected according to relevant references in the literature.

Fig. 2. Effect size of stress-induced increases in VMR on day 2. Overall effect of stress was determined by calculating the area under the curve (AUC) of the raw EMG amplitude as a function of pressure before and 24 h after stress for each animal. From these values, the % change in AUC was calculated by taking the difference between the AUC before and 24 h after stress, dividing by the AUC before stress, and multiplying by 100. All animals that received vehicle treatment were included in this analysis. Data are frequency histograms of the % increase in AUC on day 2.

Fig. 1. A: effect of 1-h water avoidance (WA) stress on visceromotor response (VMR) to graded, phasic colorectal distension (CRD) immediately after WA and on day 2 (n = 12). Data were normalized as percentage of baseline of averaged response at 60 mmHg in the ascending series. There was a significant increase in VMR 24 h after stress compared with baseline (P < 0.01). B: effect of 1-h sham stress on VMR immediately and 24 h after the procedure (n = 12). There was no change in VMR after sham stress. C: mean changes in electromyographic (EMG) amplitude in stressed and sham-stressed animals. The mean baseline EMG amplitude (in percentage of baseline) was subtracted from the day 2 mean EMG amplitude at each pressure step, in each group. These mean changes were compared between the two groups by using one-way ANOVA and post hoc t-tests. Data are presented as mean change ± SE of difference. *P < 0.05 compared with sham stress. There is a significant difference between stressed and sham-stressed animals at 40 and 60 mmHg.
Fig. 3. Mean changes in EMG amplitude between baseline and VMR on day 2 in drug-treated vs. vehicle-treated animals. The mean baseline EMG amplitude (in percentage of baseline) was subtracted from the day 2 mean EMG amplitude at each pressure step in each group. These mean changes were compared between the 2 groups by using ANOVA and post hoc t-tests. Data are presented as mean change ± SE of difference. *P < 0.05 compared with vehicle at the particular pressure. A: CP-154526 (32 mg/kg) administered before WA (n = 12 rats) significantly decreased the VMR on day 2 compared with vehicle (n = 11 rats) at 40 and 60 mmHg. B: CP-154526 (32 mg/kg) administered before the CRD on day 2 (n = 12 rats) significantly decreased VMR on day 2 compared with vehicle (n = 11 rats) at 40 mmHg. At 60 mmHg, the decrease in VMR almost reached significance (P = 0.07). **P = 0.07 compared with vehicle. C: RP-67580 (3 or 1 mg/kg, n = 10 in each group) injected before CRD on day 2 significantly decreased VMR 24 h after stress compared with vehicle (n = 10 rats) at 40 and 60 mmHg. SR-140333 (1 mg/kg) injected before CRD on day 2 (n = 10 rats) significantly decreased VMR on day 2 compared with vehicle (n = 10 rats) at 40 and 60 mmHg. At 0.1 mg/kg, there was no significant change in VMR 24 h after stress compared with vehicle.

Table 1. Effect of RP-67580 injected before WA on VMR on day 2

<table>
<thead>
<tr>
<th>mmHg</th>
<th>Baseline</th>
<th>Day 2*</th>
<th>Mean change</th>
<th>Baseline</th>
<th>Day 2*</th>
<th>Mean change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.1±1.9</td>
<td>4.1±1.2</td>
<td>−1.0±2.3</td>
<td>2.5±0.8</td>
<td>11.0±6.7</td>
<td>8.4±6.8</td>
</tr>
<tr>
<td>20</td>
<td>9.1±2.5</td>
<td>44.9±18.2</td>
<td>35.8±18.3</td>
<td>13.0±6.4</td>
<td>22.9±6.8</td>
<td>9.9±9.4</td>
</tr>
<tr>
<td>40</td>
<td>59.0±9.4</td>
<td>83.4±24.1</td>
<td>24.4±25.9</td>
<td>37.9±6.3</td>
<td>61.3±7.4</td>
<td>23.4±9.7</td>
</tr>
<tr>
<td>60</td>
<td>100±0</td>
<td>132.2±25.0</td>
<td>32.2±25.0</td>
<td>100±0</td>
<td>124.6±5.0</td>
<td>24.6±5.0</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE and mean change ± SE of difference. Electromyographic (EMG) amplitude percentage of baseline at 10, 20, 40, and 60 mmHg at baseline, percentage on day 2, and mean change between baseline and day 2 for rats treated with RP-67580 (3 mg/kg) before WA or vehicle (n = 10 in both groups) are shown. WA, water avoidance; VMR, viseromotor response. *P < 0.05 compared with baseline.
Data Analysis and Statistics

EMG amplitudes were normalized as percent of baseline to the averaged response at 60 mmHg in the ascending series. The effect of stress on the VMR to CRD was analyzed by comparing the poststress measurements with the baseline within the same group of animals. Data were analyzed by repeated-measures ANOVA followed by Tukey’s postcomparison test. The overall effect of stress was determined by calculating the AUC of the raw EMG amplitude as a function of pressure before and after stress for each animal. From these values, the percent change in AUC was calculated by taking the difference between the AUC before and after stress, dividing by the AUC before stress, and multiplying by 100. The effect of drug treatment on the poststress VMR was assessed comparing the mean changes from baseline between treatment groups by using one-way ANOVA and post hoc t-tests. P < 0.05 was considered significant.

RESULTS

Effect of Stress on VMR

No significant change in the VMR was observed (Fig. 1A) immediately after the hour of WA stress; however, a significant increase in the VMR developed 24 h after the WA stress (P < 0.01 compared with baseline, repeated-measures ANOVA, n = 12) reflected by a mean increase in AUC of 69.7 ± 13.1%. To confirm that the observed increase in VMR to CRD is due to the earlier experience of stress rather than being related to other factors (such as repeated distensions or conditioned responses to the site of CRD), the influence of repeated CRD on two consecutive days without any psychological intervention, as well as 1-h sham WA (dry tanks) were evaluated. Repeated CRDs on two consecutive days without any stress intervention did not lead to a significant increase in VMR (data not shown). Sham WA had no effect on VMR immediately after the procedure or 24 h later (Fig. 1B). Comparison of the mean change from baseline 24 h after stress revealed that there was a significant increase in VMR in the stressed group at 40 mmHg (P < 0.05) and 60 mmHg (P < 0.0001) compared with sham stressed animals (Fig. 1C).

Effect Size and Variability of SICH

Interanimal variability in delayed SICH was assessed by calculating the percent change in AUC for the VMR. All rats that received vehicle treatment for the experiments described above were included. A total number of 102 rats were used in this analysis. The mean increase in the VMR was 47 ± 5%. Eighty-six percent of animals exposed to WA (88 of 102) showed increases in AUC 24 h after the stress (Fig. 2) with 47% (48 of 102) exhibiting increases between 1 and 50% over baseline and 39% (40 of 102) showing increases in AUC of >51% over baseline.

Effect of CRF\(_i\)R Antagonist CP-154526

The CRF\(_i\)R-selective antagonist CP-154526 was used in a dose previously found to be effective in blocking SICH in a different rat model (42). The compound was administered subcutaneously either 45 min before WA or 1 h before the start of CRD on day 2 to evaluate the dependence of delayed SICH on peripheral and/or central CRF\(_i\)Rs. Administration before WA (n = 12) resulted in a significant decrease in the VMR on day 2 at 40 mmHg (P < 0.05) and 60 mmHg (P < 0.001) compared with the vehicle group (n = 11, Fig. 3A). Similarly, injection before the start of the CRD on day 2 (n = 12) significantly decreased VMR on day 2 at 40 mmHg (P = 0.02), At 60 mmHg, a decrease in VMR was observed but did not achieve significance (P = 0.07, Fig. 3B). In both vehicle groups, stress significantly increased VMR on day 2 (P < 0.05 compared with baseline). The effect of the compound on sham stressed animals was assessed in a separate group of animals (n = 10 per group). Stress did not lead to an increase in colonic sensitivity on day 2 compared with baseline in any of the groups. Injection of CP-154526 before the sham procedure or before the CRD on day 2 did not change VMR to CRD at 24 h (data not shown), suggesting that CP-154526 does not have an effect on the VMR in nonstressed animals.

Effect of NK\(_i\)R Antagonists RP-67580 and SR-140333

Doses of NK\(_i\)R-selective antagonist RP-67580 were selected according to relevant references in the literature (16, 38). The compound was administered 15 min before the WA stress to evaluate the dependence of delayed SICH on NK\(_i\)Rs. RP-67580 (3 mg/kg) given before WA stress on day 1 did not decrease the VMR assessed on day 2 (Table 1). In both the vehicle and the treated group (n = 9 in each group), stress significantly increased VMR on day 2 (P < 0.05 compared with baseline). In contrast, injection of RP-67580 at 3 mg/kg before the CRD on day 2 resulted in a significant decrease in VMR on day 2 compared with vehicle (at 40 mmHg, P < 0.01; at 60 mmHg, P < 0.0001; Fig. 3C). When the dose was reduced to 1 mg/kg, RP-67580 still significantly decreased VMR on day 2 compared with vehicle (at 40 mmHg, P = 0.01; at 60 mmHg, P < 0.001; Fig. 3C). In the vehicle group (n = 12), stress significantly increased VMR on day 2 compared with baseline (P < 0.05). Sham stress did not lead to an increase in colonic sensitivity on day 2 compared with baseline. Administration of RP-67580 (3 mg/kg) before the CRD

Table 2. Effect of SR-140333 injected before WA on VMR on day 2

<table>
<thead>
<tr>
<th>mmHg</th>
<th>SR-140333</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 2*</td>
</tr>
<tr>
<td>10</td>
<td>3.6±0.8</td>
<td>1.7±0.7</td>
</tr>
<tr>
<td>20</td>
<td>8.4±1.8</td>
<td>13.1±3.4</td>
</tr>
<tr>
<td>40</td>
<td>31.9±4.1</td>
<td>66.1±8.1</td>
</tr>
<tr>
<td>60</td>
<td>100±0</td>
<td>130.2±8.4</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE and mean change ± SE of difference. EMG amplitude percentage of baseline at 10, 20, 40, and 60 mmHg at baseline, percentage on day 2, and mean change between baseline and day 2 for rats treated with SR-140333 (1 mg/kg) before WA or vehicle (n = 10 in both groups) are shown. *P < 0.05 compared with baseline.
on day 2 in sham stressed animals decreased the VMR on day 2 below baseline (data not shown), indicating that RP-67580 may have an analgesic effect in this model.

To confirm our findings with RP-67580 and rule out that the observed effects may be related to nonspecific effects of this compound, we repeated the experiments by using a different NK₁R antagonist, SR-140333. The compound was used in a dose previously shown to be effective in a model of SICH (2). Administration of SR-140333 before WA (1 mg/kg) did not change VMR on day 2 compared with vehicle (Table 2). Stress significantly increased the VMR on day 2 in both the vehicle and treated groups (P < 0.05 compared with baseline). In contrast, injection of 1 mg/kg before the CRD on day 2 resulted in a significant decrease in VMR on day 2 compared with vehicle (at 40 mmHg, P < 0.05; at 60 mmHg, P < 0.001; n = 10 in both groups, Fig. 3D). Treatment with SR-140333 at a dose of 0.1 mg/kg before the CRD on day 2 did not change VMR on day 2 significantly (Fig. 3D). In a separate group of animals (n = 10), the per se effect of the compound was assessed on sham-stressed animals. Sham stress did not lead to an increase in colonic sensitivity on day 2 compared with baseline, and the administration of SR-140333 (1 mg/kg) before the 24-h measurement in sham-stressed animals did not influence the VMR on day 2 (data not shown).

**Chemical Sympathectomy**

We first determined whether the repeated treatment of the animals with 6-OHDA successfully ablated the sympathetic innervation of the gut wall by evaluating the immunohistochemical labeling of NPY, a neuropeptide colocalized in noradrenergic nerve terminals innervating the gut mucosa (12, 32). In control animals, NPY immunostaining was specifically localized to neural elements within the submucosa and muscularis layers of the colon (Fig. 4A). Rats treated with chemical sympathectomy demonstrated a markedly reduced amount and intensity of labeling compared with control treatment (Fig. 4B). Nonspecific labeling within the mucosa was present (Fig. 4C) but did not interfere with the interpretation of the specific NPY peptide immunolocalization and assessment of the changes during chemical sympathectomy. These data confirmed that chemical sympathectomy resulted in loss of NPY-containing neural components within the colonic wall.

Both the 6-OHDA- and vehicle-treated groups exhibited increased VMR 24 h after stress (P < 0.05 compared with baseline, n = 10 in each group, Table 3). There was no significant difference between the two groups when mean changes from baseline were compared.

**DISCUSSION**

This study in male Wistar rats demonstrates that 1-h WA, an acute psychological stressor, does not have an immediate effect on colonic nociception but results in the delayed development of colonic hypersensitivity 24 h after the stress procedure. The development of this delayed SICH requires activation of CRF₁Rs, but not NK₁Rs, at the time of the initial stressor. In contrast, the expression of the delayed colonic hypersensitivity is dependent on activation of both CRF₁Rs and NK₁Rs. Furthermore, the development and expression of the delayed hypersensitivity is not dependent on effects mediated by peripheral noradrenergic nerves.

**Delayed SICH**

In the present study, exposure to 1-h WA stress resulted in delayed SICH in 84% of all animals tested, with an average increase in the nociceptive response over baseline of 47%. Thus SICH, in this model, was both robust and inducible in a reliable fashion. The increase in colonic sensitivity 24 h after WA was only observed at noxious intensities of CRD (40 and 60 mmHg) compared with the sham stressed controls, consist-
tent with the definition of hyperalgesia (increased nociceptive response to stimuli of noxious intensity). Although evidence for SICH has previously been reported (8, 15), to our knowledge this is the first study to demonstrate a delayed effect of an acute stressor on visceral sensitivity 24 h later. The observed increase in colonic sensitivity may not be due to the earlier experience of WA stress but rather represent a conditioned fear response to the CRD testing environment. Alternatively, it may represent nonspecific changes in colonic sensitivity due to repeated testing. To rule out these confounding factors, we performed two control experiments: 1) measurement of the stability of the nociceptive response to repeated CRDs on two consecutive days without any intervention, and 2) the effect of a 1-h sham WA stress in which the rats were exposed to the WA tanks without water. Neither of these interventions showed any effect on the nociceptive response on day 2, indicating that delayed SICH is a specific stress-related phenomenon.

**Dependence of Delayed SICH on Receptors for CRF and SP**

Mechanism(s) underlying SICH are incompletely understood and may involve both central and peripheral components (30). Preliminary evidence suggests an involvement of central CRF release and activation of CRF1Rs (42). In the present study, peripheral administration of the selective CRF1R antagonist CP-154526, which is capable of passing through the blood-brain barrier (21), before the WA stress prevented delayed SICH. The dose of the antagonist was chosen on the basis of the ability of the compound to prevent SICH in a different stress model (42). This observation suggests that activation of CRF1Rs on the day of the stressor is a necessary condition for the development of subsequent colonic hypersensitivity. In contrast, inhibition of NK1Rs had no effect on the development of delayed SICH. One interpretation of these findings is that stress-induced CRF1R activation results in an upregulation of this system by day 2, resulting in an enhanced central stress response to the visceral pain stimulus (37). Thus inhibition of CRF1R activation induced by WA stress could prevent CRF and CRF1R upregulation and thereby the development of delayed SICH. Consistent with this hypothesis is also the observation that the delayed colonic hypersensitivity was blocked by administration of CRF1R antagonist before CRD on day 2 of the study.

In contrast to the effect of CRF1R antagonism, inhibition of the NK1R was only effective in blocking the expression of the delayed SICH. A possible concern regarding the use of existing NK1R antagonists is their specificity. Several different compounds have been reported to have nonspecific analgesic effects on neuronal responses, possibly via inhibitory effects on voltage-sensitive Ca2+ channels. Both doses of RP-67580 decreased the VML 24 h after stress as well as sham stress below baseline, suggesting an analgesic rather than an antihyperalgesic effect of the compound in our model. Prior studies have demonstrated that RP-67580 has potent analgesic activity similar to morphine in response to subcutaneous formalin injection and the phenylbenzoxazine-induced writhing test (13). In contrast, SR-140333 abolished only stress-induced hypersensitivity but did not have an effect on baseline or in sham-stressed animals. The dose of the antagonist used in this study was based on previous reports (2) of its effectiveness in a model of SICH. These observations suggest that SR-140333 does not have analgesic properties and that the observed effects are mediated by inhibition of NK1Rs.

The ability of both CRF1R and NK1R antagonists to prevent delayed SICH suggests two intriguing possibilities: 1) activation of the CRF1R system resulted both in its own upregulation and in the expression of NK1Rs involved in SICH; and 2) antagonism of both receptor systems blocked the expression of delayed SICH, which is consistent with the hypothesis that the CRF/CRF1R and SP/NK1R systems are operating “in series.” Upregulation of the CRF and SP systems could result in colonic hypersensitivity regardless of whether these changes occurred peripherally, centrally, or at both locations. Our experiments were not aimed at locating the site of action of the CRF/CRF1R and SP/NK1R systems in this model. Future studies will be needed to address this important question.

**Role of the SNS in Delayed SICH**

Stress-induced activation of the SNS could play a role in the development of SICH by several mechanisms including direct or indirect sensitization of primary afferent nerve terminals. Sympathetically maintained pain is a well-described mecha-

### Table 3. Effect of chemical sympathectomy on VMR on day 2

<table>
<thead>
<tr>
<th>mmHg</th>
<th>Chemical Sympathectomy</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 2*</td>
</tr>
<tr>
<td>10</td>
<td>4.2±0.6</td>
<td>11.9±2.6</td>
</tr>
<tr>
<td>20</td>
<td>19.7±2.3</td>
<td>46.2±7.4</td>
</tr>
<tr>
<td>40</td>
<td>62.4±7.9</td>
<td>84.7±4.9</td>
</tr>
<tr>
<td>60</td>
<td>100±0</td>
<td>125.4±7.6</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE of displacement. EMG amplitude percentage of baseline at 10, 20, 40, and 60 mmHg at baseline, percentage day 2, and mean change between baseline and day 2 for rats treated with 6-hydroxydopamine or vehicle (n = 10 rats in each group) are shown. *P < 0.05 compared with baseline.
nism for some forms of chronic pain (19). The SNS is one arm of the stress response that has been shown to modulate peripheral immune function. For example, mast cell degranulation can be induced by neuronal stimulation of sympathetic ganglia (22). To evaluate a possible role of the peripheral sympathetic innervation of the colon in the development of delayed colonic hypersensitivity, we performed the same experiments with WA stress and CRD after a chemical sympathectomy with 6-OHDA. Completeness of the chemical sympathectomy was verified by performing immunohistochemistry for NPY in the colonic submucosa, a peptide colocalized with norepinephrine in a subset of noradrenergic nerves (12). Disappearance of NPY immunoreactivity in the gut mucosa has previously been used to document the effectiveness of chemical sympathectomy (32). Because chemical sympathectomy did not affect the development of SICH, it is unlikely that release of norepinephrine (or NPY) from postganglionic sympathetic nerves plays a role in the development of SICH in this model. A possible role of other peripheral stress mediators, such as corticosterone or epinephrine, which have been shown to sensitize primary afferents in a model of mechanical hyperalgesia (23), was not evaluated in this study.

In summary, we demonstrate that male Wistar rats develop delayed colonic hypersensitivity after a relatively mild psychological stressor. This stress sensitization appears to involve upregulation of both the CRF/CRF$_1$R and SP/NK$_1$R systems. Increased cerebral spinal fluid levels of SP and CRF have been reported in various stress-sensitive disorders, including posttraumatic stress syndrome (3), and preliminary data from our group suggest increased cerebrospinal fluid levels of these neuropeptides in the majority of female IBS patients (11). Thus upregulation of these systems may play a role in mediating recurrent abdominal pain and discomfort in a subset of these patients.

ACKNOWLEDGMENTS

The authors thank Dr. Jeffrey Gornbein, Department of Biostatistics, University of California, Los Angeles, CA, for assistance in the statistical analysis of the data.

DISCLOSURE

The CURE Antibody/Radioimmunoassay and Center for Neurovisceral Sciences (CNS) Neuroassay/Immunohistochemistry Core Laboratories were utilized in performing the immunohistochemistry experiments described in this paper.

GRANTS

This study was supported by AstraZeneca R&D, Moelndal, Sweden, National Institute of Diabetes and Digestive and Kidney Diseases Grant 1P50-DK-64539-01 (to CNS and Women's Health) and P30-DK-41301 (to CURE/University of California Los Angeles Digestive Disease Research).

REFERENCES

2. Bradeli S, Eutamene H, García-Villar R, Fioramonti J, and Bueno L. Stress-induced visceral hypersensitivity in female rats is estrogen-depen-
13. Garret C, Carruette A, Fardin V, Moussaoui S, Peyronel JF, Blan-
19. Jänig W and Habler HJ. Specificity in the organization of the autonomic nervous system: a basis for precise neural regulation of homeostatic and protective body functions. In: The Biological Basis for Mind Body Inter-
21. Keller C, Bruehlauer A, Lemaire M, and Enz A. Brain pharmacoki-
23. Khasar SG, McCarter G, and Levine JD. Epinephrine produces a β-adrenergic receptor-mediated mechanical hyperalgesia and in vitro sensi-

AJP-Gastrointest Liver Physiol • VOL 286 • APRIL 2004 • WWW.AJPGI.ORG