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Luminal hypertonicity and acidity modulate colorectal afferents and induce persistent visceral hypersensitivity

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La JH, Feng B, Schwartz ES, Brumovsky PR, Gebhart GF. Luminal hypertonicity and acidity modulate colorectal afferents and induce persistent visceral hypersensitivity. *Am J Physiol Gastrointest Liver Physiol* 303: G802–G809, 2012. First published August 1, 2012; doi:10.1152/ajpgi.00259.2012.—Carbohydrate malabsorption such as in lactose intolerance or enteric infection causes symptoms that include abdominal pain. Because this digestive disorder increases intracolonic osmolarity and acidity by accumulation of undigested carbohydrates and fermented products, we tested whether these two factors (hypertonicity and acidity) would modulate colorectal afferents in association with colorectal nociception and hypersensitivity. In mouse colorectum-pelvic nerve preparations in vitro, afferent activities were monitored after application of acidic hypertonic saline (AHS; pH 6.0, 800 mosM). In other experiments, AHS was instilled intracolonically to mice and behavioral responses to colorectal distension (CRD) measured. Application of AHS in vitro excited 80% of serosal and 42% of mechanically-insensitive colorectal afferents (MIAs), sensitizing a proportion of MIAs to become mechanically sensitive and reversibly inhibiting stretch-sensitive afferents. Acute intracolonic AHS significantly increased expression of the neuronal activation marker pERK in colon sensory neurons and augmented noxious CRD-induced behavioral responses. After three consecutive daily intracolonic AHS treatments, mice were hypersensitive to CRD 4–15 days after the first treatment. In complementary single fiber recordings in vitro, the proportion of serosal class afferents increased at *day 4*; the proportion of MIAs decreased, and muscular class stretch-sensitive afferents were sensitized at *days 11–15* in mice receiving AHS. These results indicate that luminal hypertonicity and acidity, two outcomes of carbohydrate malabsorption, can induce colorectal hypersensitivity to distension by altering the excitability and relative proportions of colorectal afferents, suggesting the potential involvement of these factors in the development of abdominal pain.

carbohydrate malabsorption; intraluminal hypertonicity; intraluminal acidity; colorectal distension

GASTROINTESTINAL CARBOHYDRATE malabsorption (CM) results from imperfect enzymatic breakdown of dietary carbohydrates, improper transepithelial transport of monosaccharides, or damage to the epithelial surface of the small intestine associated with, for example, an enteric infection (3). This digestive disorder causes an array of gastrointestinal symptoms including abdominal discomfort/pain, osmotic diarrhea, and flatulence, which are thought to be due to accumulation of unabsorbed sugars that are eventually fermented by microflora to further produce gas, increase the osmotic load, and decrease pH inside the colon lumen. Regarding abdominal discomfort/pain, it has not been systemically studied whether these altered intracolonic conditions (hypertonicity and acidity) affect the physiology of visceral sensory nerves.

The foregoing is of interest because there is overlap between CM and irritable bowel syndrome (IBS) with respect to the nature of symptoms and etiology. Bloating, flatulence, abdominal discomfort/pain and altered bowel habits (diarrhea or constipation) are common complaints of IBS patients, a significant proportion of which develop IBS after an enteric infection (postinfectious IBS) that could have included epithelial damage (10, 29). Although it has been proposed that CM and IBS may be unrelated disorders based on the comparable prevalence of CM in IBS patients and in healthy subjects (11, 15), considerable ethnic and regional variances (2, 27, 32) do not permit a conclusion at present.

Postinfectious IBS develops after resolution of the initial insult in 4–31% of infected subjects (16). Considering that certain enteric infections cause CM [e.g., 17% of *Campylobacter jejuni*-infected subjects and 35% of *Escherichia coli*-infected subjects exhibit osmotic diarrhea, respectively (7)], one could hypothesize that CM-induced changes in intracolonic conditions (hypertonicity and acidity) may cause persistent changes in the colorectal sensory system, which can be expressed as IBS-like symptoms. To test this hypothesis, we first examined the effects of intracolonic hypertonicity and acidity, the two outcomes of CM, on the physiology of sensory nerves innervating the colorectum, and secondly whether the two factors could induce chronic abdominal discomfort/pain as in IBS. Here we report that hypertonicity and acidity modulate the activities of colorectal afferents with distinguishable modalities, and induce a weeks-long hypersensitivity to colorectal distension in the absence of structural changes in mouse colon. Portions of these data have been reported in abstract form (22, 23).

MATERIALS AND METHODS

Animals. Adult male C57BL/6 (Jackson Laboratory, Bar Harbor, ME) mice (25–30 g), housed under a 12:12-h light-dark cycle in an AAALAC-accredited facility, were used throughout. Water and food were provided ad libitum. All procedures were approved by and in accordance with the guidelines of the Institutional Animal Care and Use Committee, University of Pittsburgh.

In vitro colorectal afferent recording. Mice were euthanized by CO_2 inhalation and were subsequently exsanguinated by cardiac perforation. The distal colorectum with attached pelvic nerve was opened longitudinally along the mesenteric border, pinned flat mucosal side up in a tissue chamber filled with Krebs solution [in mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4(H_2O)_7$, 2.5 $CaCl_2$, 11.1 D-glucose, 2 butyrate, and 20 acetate, 0.004 nifedipine

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and 0.003 indomethacin], and the pelvic nerve teased into fine bundles for single-fiber electrophysiological recordings.

Colorectal afferent endings were located by electrical stimulation (0.5 ms duration, 0.3 Hz) and subsequently tested for mechanosensitivity. Afferents were classified as serosal, muscular, mucosal, muscular/mucosal, or mechanically-insensitive colorectal afferents (MIAs) by probing with von Frey-like nylon monofilaments (0.4–1.4 g force), mucosal stroking with a nylon filament (10 mg force), and circumferential stretch (0–170 mN ramp, 5 mN/s) as previously reported (13). Afferent activity was filtered (0.3–10 kHz), amplified (model DAM 80; World Precision Instruments, New Haven, CT), digitized at 20 kHz (model 1401 interface; CED, Cambridge, UK), and analyzed using Spike2 software (CED).

Electrode implantation for in vivo recordings of visceral nociception. A pair of sterile wire electrodes was surgically implanted (2% isoflurane for maintenance; Hospira, Lake Forest, IL) into the external oblique abdominal musculature with the tips of other ends exposed at the back of the neck for electromyographic recordings to quantify nociceptive responses to colorectal distension (CRD). Mice were not used for at least 5 days after electrode implantation.

CRD. A 2-cm-long balloon was inserted into the colorectum 1 cm proximal from the anus under 3% isoflurane and connected to a distension device. The mouse was placed in a plastic cylinder to restrain its movement and allowed to recover from isoflurane exposure for 30 min. In one set of experiments, noxious CRD (60 mmHg, 10 s duration, 4 min interstimulus interval) was delivered to evoke a visceromotor response (VMR) before (baseline) and after intracolonic treatments. In another set of experiments, baseline VMRs to an ascending series of graded CRD (15, 30, 45, and 60 mmHg for 10 s every 4 min, 3 repetitions at each intensity) were measured. To study acute effects of acidic hypertonic saline (AHS) on VMRs to graded CRD, 2 days after baseline VMR measurement, mice were treated with either intracolonic saline or AHS 8 min before the start of an ascending series of CRD. Effects of repeated AHS application were studied by applying intracolonic saline or AHS once daily for three consecutive days and measuring VMRs to graded CRD 4, 8, and 15 days after the first treatment. VMRs were normalized to the maximum baseline response.

Intracolonic treatment. Under 3% isoflurane, 0.15 ml of control saline (in mM: 140 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 3 $CaCl_2$ and 10 D-glucose, pH 7.4, 300 mosM), acidified hypertonic saline (AHS, pH 6.0, 800 mosM by adding D-mannitol; Sigma-Aldrich, St. Louis, MO) or 2,4,6-trinitrobenzene sulfonic acid (TNBS; 10 mg/ml, Sigma-Aldrich, dissolved in 50% ethanol) was instilled inside the colorectal lumen transanally via a 22-gauge feeding needle. In experiments where intracolonic treatments were performed between or immediately before CRD testing, a balloon-catheter was used to instill test solutions intracolonically without removing the balloon.

Labeling of colon sensory neurons. The distal colon was surgically exposed under 2% isoflurane for maintenance of anesthesia. The retrograde tracer Fast Blue (FB; 1% in sterile saline; EMS-Chemie, Gross-Umstadt, Germany) was injected into the distal colon wall using a microsyringe (3–5 sites, each in a volume of ~3 μl). Mice were used for experiments 3–4 wk after FB injection.

Immunostaining. Mice were euthanized by CO_2 inhalation 3 min after intracolonic AHS instillation, transcardially perfused with ice-cold Lana's fixative (4% paraformaldehyde, 14% picric acid in 0.4 M phosphate buffer) and L6 dorsal root ganglia (DRG) harvested bilaterally. After cryoprotection in 20% sucrose, fixed tissue was embedded in OCT compound (Sakura Finetek, Japan), frozen, and sectioned at 14 μm thickness. Tissue sections from all experimental groups were mounted on the same slides, incubated with rabbit anti-pERK [phosphorylated extracellular signal-regulated kinase 1/2 (pERK); 1:600; Cell Signaling, Danvers, MA] overnight at 4°C and then with Cy3-conjugated anti-rabbit IgG (1:200; Jackson Immunorsearch, West Grove, PA) for 1 h at room temperature. Processed tissue sections were photographed using a microscope-mounted digital camera (model DFC340FX; Leica Microsystems, Bannockburn, IL) and immunostained FB-labeled neurons were counted after setting the threshold of eight-bit grey scale images to exclude at least 99.99% of background intensity based on its Gaussian distribution using ImageJ (version 1.42q, NIH).

Myeloperoxidase activity assay. Mice were overdosed with inhaled isoflurane and distal colons were dissected, weighed, added to a beaker containing 1.0 ml 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich), minced and sonicated for 10 s before homogenization for 30 s. The samples underwent three freeze-thaw cycles and were centrifuged twice, reacted with *O*-dianisidine dihydrochloride (Sigma-Aldrich), and read on a plate reader at 460 nm.

Data analyses. Data were expressed as means ± SE with n, the number of samples and N, the number of mice. Fisher's exact test (FET) was used for analysis of 2×2 contingency tables. Student's t-test was used to compare means of two groups. One- or two-way ANOVA for repeated measures (RM) with post hoc multiple comparison tests (Bonferroni's or Dunnett's) was used for more than two groups. Results were considered statistically significant when $P < 0.05$.

RESULTS

Effects of AHS on colorectal afferents in vitro. In lactose intolerance and intestinal inflammation, stool pH can be lower than pH 6.0 and stool osmolarity as high as 490 (lactose intolerance) or 610 mosM (inflammation) (8). Based on the reported stool properties in CM patients, we prepared an AHS solution, adjusting pH and osmolarity to pH 6.0 and 500 or 800 mosM, respectively.

Sensory (afferent) fibers in the pelvic nerve are categorized into five different classes based on their response properties: four mechanosensitive classes that respond to blunt probing (serosal that respond only to blunt probing, mucosal that respond also to mucosal stroking, muscular that also respond to circumferential colon wall stretch, and muscular/mucosal that also respond to both wall stretch and mucosal stroking) and a mechanically insensitive afferent (MIAs) class, the nomenclature of which does not necessarily reflect histological location of their endings (5, 13). As an initial step in evaluating whether hypertonicity and acidity affect colorectal afferent activity or responses to mechanical stimuli, we screened effects of AHS on colorectal endings representative of each class of afferent. When applied to the luminal side of receptive ending for 5 min, AHS at 800 mosM induced spontaneous action potential firing in 80% of serosal afferents ($n = 8$ of 10, $N = 7$, Fig. 1*A*) and 42% of MIAs ($n = 3$ of 7, $N = 6$, Fig. 1*B*). These afferents discharged action potentials throughout the AHS application with no apparent adaptation, and the excitatory effect of AHS was not immediately washed out after the removal of AHS from the receptive endings. Interestingly, one of the three responsive MIAs as well as two others that did not respond directly to AHS application became mechanically sensitive and responded to blunt probing after AHS application (like serosal afferents).

When acidic saline (pH 6.0, 300 mosM) and hypertonic saline (pH 7.4, 800 mosM) were applied separately to serosal and MIA endings, acidic saline and hypertonic saline also induced action potential firing but to a lesser degree than AHS, which was apparent in the after-discharge phase (Fig. 1, *A* and *B*). Likewise, AHS at 500 mosM induced weaker responses than AHS at 800 mosM; in one example, a serosal afferent fired action potentials only after exposure to AHS at 800

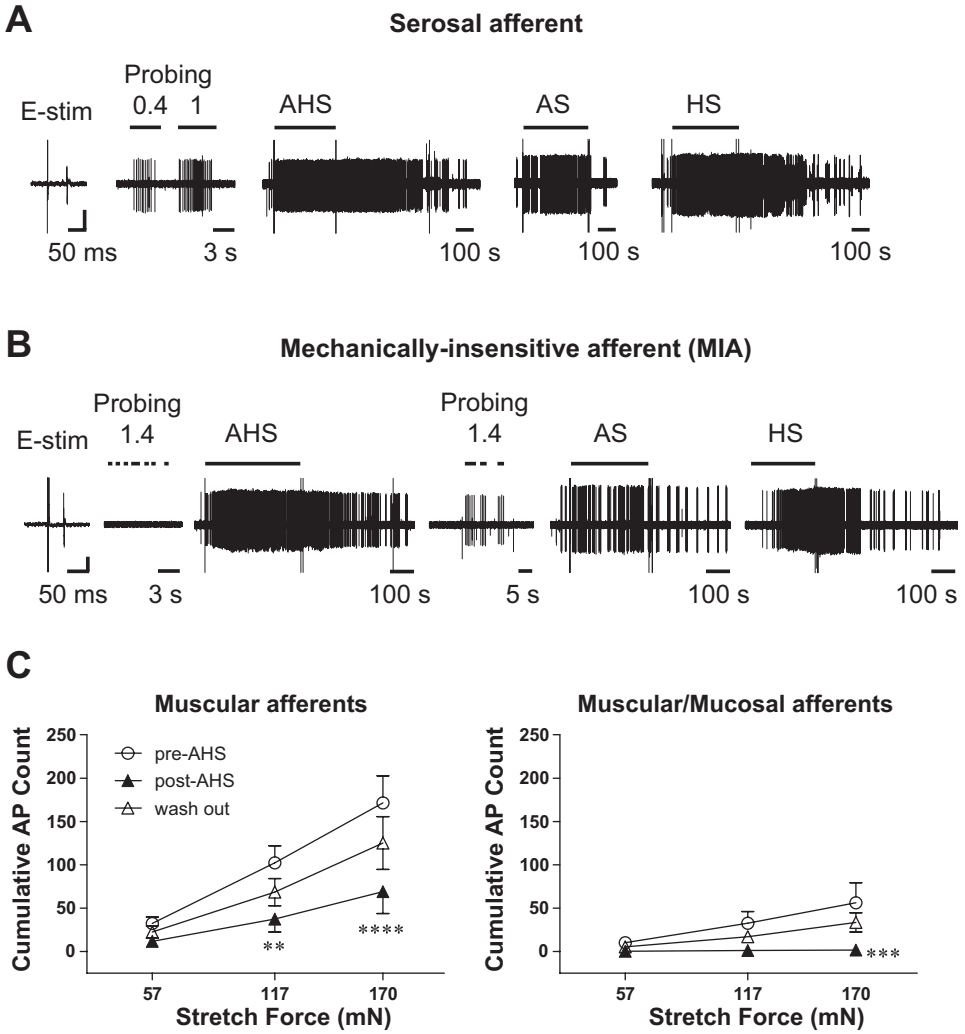

Fig. 1. Effects of acidic hypertonic saline (AHS) on colorectal afferent activity. Representative traces showing activation of a serosal afferent (*A*) and a mechanically-insensitive afferent (MIAs) (*B*) in response to AHS (pH 6.0, 800 mosM), acidified saline (AS; pH 6.0, 300 mosM) and hypertonic saline (HS; pH 7.4, 800 mosM). AHS induced the greatest response in both afferent classes. Note that the MIA acquired mechanosensitivity and responded to blunt probing after AHS application (*B*, Probing 1.4 g after AHS). Vertical bars in *A* and *B* represent 200 μV. E-stim, electrical search stimulation. *C*: stretch-encoding of muscular (*left*, $n = 9$, $N = 5$) and muscular/mucosal afferents (*right*, $n = 8$, $N = 6$). Immediately after AHS application, their stretch-encoding function was significantly but reversibly inhibited. AP, action potentials. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. pre-AHS by 2-way repeated-measures (RM) ANOVA followed by Bonferroni's post hoc test.

mosM. Therefore, we used AHS at 800 mosM throughout the rest of this study.

Unlike the excitatory/sensitizing effects on serosal afferents and MIAs, AHS significantly but reversibly attenuated the stretch-sensitivity of muscular ($n = 9$, $N = 5$) and muscular/mucosal afferents ($n = 8$, $N = 6$) (Fig. 1*C*). Their mechanical thresholds for response to stretch were also increased: from 22.1 ± 5.3 mN to 70.1 ± 22.6 mN (muscular afferents; $P < 0.01$ by one-way RM ANOVA followed by Dunnett's post hoc test), which returned to 27.0 ± 8.5 mN ($n = 8$, $N = 5$) after washout for 5 min, and from 49.5 ± 10.5 mN to 116.2 ± 22.5 mN (muscular/mucosal afferents; $P < 0.001$), returning to 51.5 ± 17.1 mN ($n = 8$, $N = 6$) after washout. Responses of mucosal afferents ($n = 4$ of 8, $N = 6$) to mucosal stroking (10 mg) were also reduced by more than 15% after AHS application (data not shown).

Effects of acute intracolonic AHS instillation in vivo. To examine in vivo the net outcome of these mixed excitatory and inhibitory effects of AHS on different classes of colorectal afferents, we instilled AHS (or control saline) intracolonically and quantified expression of the neuronal activation marker pERK (9) in colon sensory neurons in L6 DRG. As shown in Fig. 2*A*, AHS significantly increased the proportion of pERK-expressing FB-labeled neurons (44.5 ± 4.3%, $N = 6$ vs. 32.2 ± 2.8% in control, $N = 5$, $P < 0.05$ by *t*-test), indicating a net excitatory effect of AHS on colorectal sensory neurons. Expression of pERK in DRG neurons is widely taken as an index of nociceptive input (9), which we verified in a positive control group that received intracolonic capsaicin (0.1%), in which the proportion of pERK-expressing FB-labeled neurons increased to 56.7 ± 8.4% ($N = 2$; Fig. 2*A*).

Because AHS decreased responses of stretch-sensitive muscular and muscular/mucosal afferents in vitro, it was also expected that acute AHS application might attenuate in vivo responses to colorectal wall stretch by balloon distension. We tested this hypothesis by measuring VMRs to repeated noxious CRD (60 mmHg, 4-min interval between each trial) before and after intracolonic AHS instillation. As illustrated in Fig. 2*B*, VMRs to CRD 4 and 8 min after AHS instillation were neither attenuated nor different from control. VMRs to CRD 12 and 16 min after AHS instillation, however, tended to be increased and greater than control, although not statistically significant. We further tested this delayed sensitizing effect of acute AHS treatment on CRD-induced VMRs by applying AHS intracolonically 8 min before the start of an ascending series of graded CRD and observed that the VMRs to CRD was significantly increased [$F_{(1,44)} = 11.4$, $P < 0.002$ by two-way RM ANOVA,

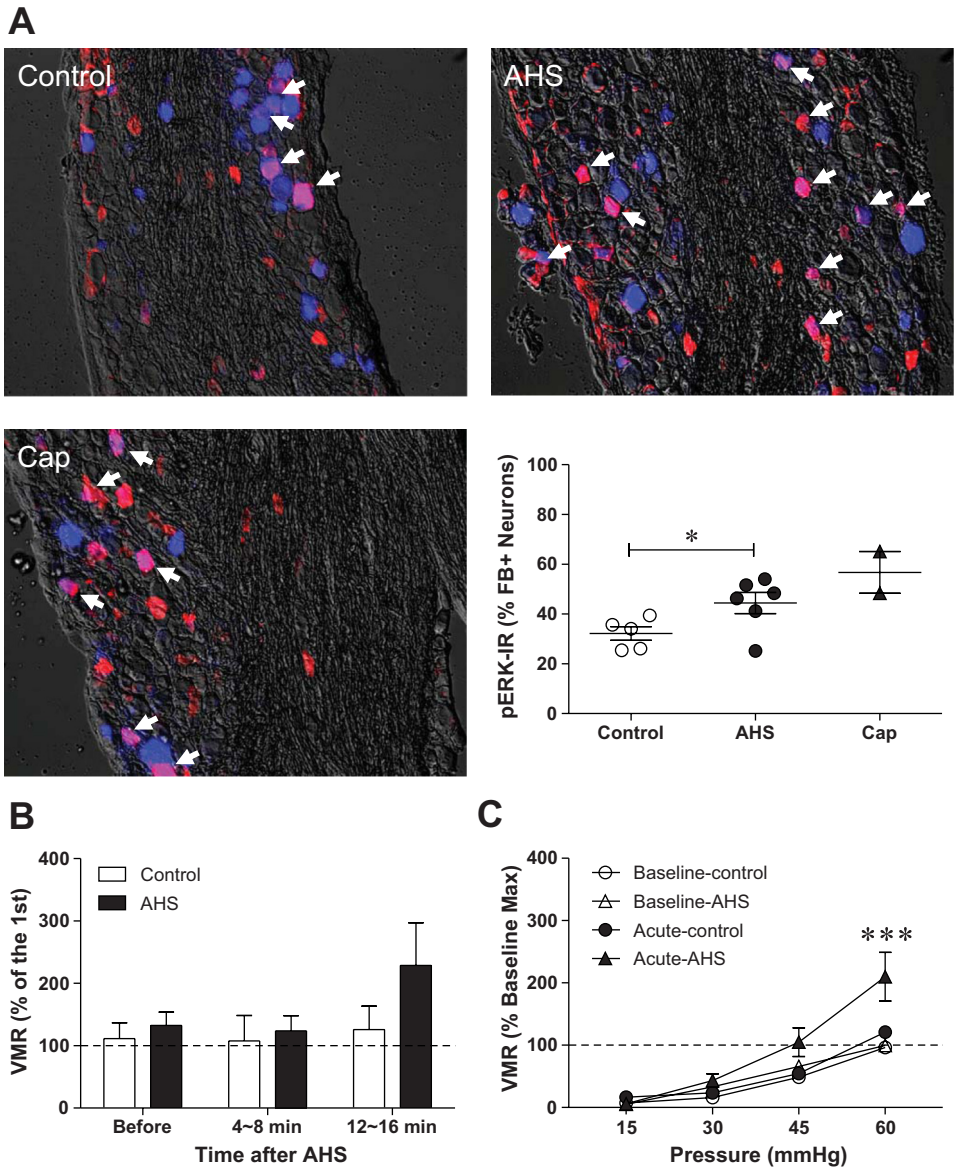

Fig. 2. Effects of intracolonic AHS on colorectal sensory responses in vivo. *A*: intracolonic AHS significantly upregulated the expression of phosphorylated extracellular signal-regulated kinses1/2 (pERK, red), a marker of neuronal excitation, in Fast Blue (FB)-labeled colon sensory neurons (blue) in L6 dorsal root ganglia (DRG). Capsaicin (Cap; 0.1%) was used as a positive control but not included in the statistical comparison. Arrows indicate DRG neurons positive to both pERK and FB (pink). $*P < 0.05$ by Student's *t*-test. *B*: visceromotor responses (VMRs) to repeated colorectal distension (CRD) at 60 mmHg before and after intracolonic AHS instillation. VMRs were normalized to the one evoked by the first 60 mmHg CRD. Two consecutive VMRs before and after (4 and 8 min; 12 and 16 min) AHS were averaged. Note the tendency toward an increase in VMRs 12~16 min after AHS instillation ($N = 6$ in each group). *C*: intracolonic AHS application 8 min before the start of an ascending series of graded CRD significantly increased the VMR. $N = 13$ in control and $N = 12$ in AHS-treated mice. $***P < 0.001$ between baseline-AHS and acute-AHS by 2-way RM ANOVA followed by Bonferroni's post hoc test.

$P < 0.001$ at 60 mmHg CRD by Bonferroni's post hoc test, Fig. 2*C*].

Effect of repeated AHS application on visceral mechanosensitivity and colorectal afferent activities. The findings described above prompted us to further examine the long-term effects of AHS on colorectal afferent endings and nociceptive behavior to CRD. It is likely that the colon lumen is repeatedly exposed to hypertonic and acidic conditions in individuals with CM. Therefore, we instilled AHS intracolonically once daily for three consecutive days. This repeated AHS application did not cause any apparent structural damage or leukocytes-based inflammation quantified as myeloperoxidase (MPO) activity in the colorectum (Fig. 3*A*); intracolonic TNBS, included as a positive control, did increase MPO activity in other mice when examined two days after intracolonic treatment.

When the VMRs to CRD was measured 4, 8, and 15 days after the first intracolonic treatment, AHS-treated mice showed approximately twofold elevated VMRs on all days tested, most apparent at noxious intensities of CRD (45 and 60 mmHg). In contrast, CRD-induced VMRs in control (saline-treated) mice were stable throughout the time course of the study (Fig. 3*B*).

To understand the sensory neurobiological aspects of this AHS-induced persistent colorectal hypersensitivity, we examined in vitro the properties of colorectal afferents in mice treated with either AHS or control saline. In pelvic nerves innervating the colorectum, we encountered serosal class afferents more frequently in mice 4 days after the first AHS application than in control ($n = 18$ of 42, $N = 4$ vs. $n = 9$ of 43, $N = 4$ in control, $P < 0.05$ by FET). Likewise, there was a tendency toward an increase in the proportion of serosal afferents 11–15 days after AHS ($n = 17$ of 47, $N = 4$ vs. $n = 8$ of 39, $N = 4$ in control) and a significant decrease in the MIA proportion ($n = 8$ of 47, $N = 4$ vs. $n = 14$ of 39, $N = 4$ in control, $P < 0.05$ by FET). Other mechanosensitive afferent classes were unchanged in their proportions between control and AHS-treated mice (Fig. 4*A*).

The stretch-encoding of muscular and muscular/mucosal afferent classes was virtually identical between control and

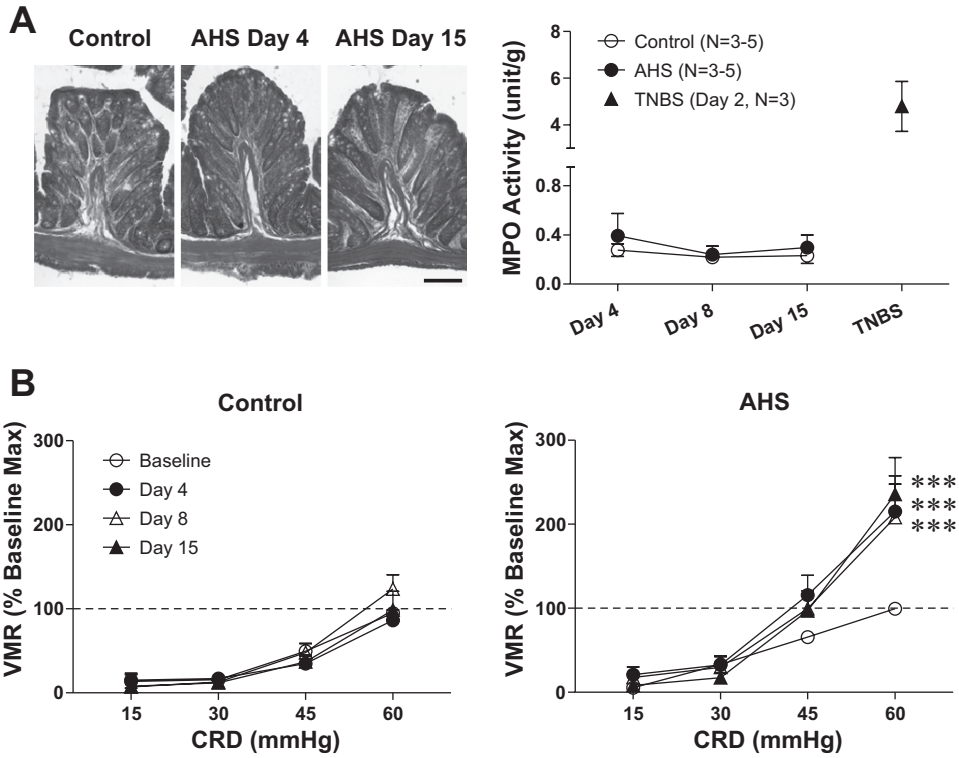

Fig. 3. Effects of AHS on colorectal structure and responses to CRD. *A*: daily intracolonic instillation of AHS for 3 consecutive days did not cause apparent histological changes (*left*, bar = 100 μm) or an increase in myeloperoxidase (MPO) activity, a marker of leukocytes-based inflammation (*right*), 4 or 15 days after the first AHS treatment. Note the increase in MPO activity in mice treated with 2,4,6-trinitrobenzene sulfonic acid (TNBS), included as a positive control. *B*: VMRs to graded CRD in control (*left*, $N = 10$) and AHS-treated mice (*right*, $N = 12$) recorded 4, 8, and 15 days after the first intracolonic AHS treatment. ***$P < 0.001$ vs. baseline by 2-way RM ANOVA followed by Bonferroni's post hoc test.

AHS-treated mice at *day 4* (Fig. 4*B*), and their response thresholds to stretch were unchanged (muscular afferents: 24.1 ± 5.9 mN, $n = 13$, $N = 5$ in control, vs. 30.4 ± 8.3 mN, $n = 11$, $N = 6$ in AHS-treated mice; muscular/mucosal afferents: 33.4 ± 7.5 mN, $n = 19$, $N = 6$ in control vs. 30.1 ± 8.7 mN, $n = 12$, $N = 4$ in AHS-treated mice).

At 11–15 days post-AHS instillation, muscular afferents exhibited increased stretch-sensitivity (Fig. 4*C*), and their response threshold to stretch was correspondingly reduced significantly (20.8 ± 2.5 mN, $n = 17$, $N = 7$ in AHS-treated mice, vs. 48.7 ± 10.2 mN, $n = 20$, $N = 7$ in control, $P < 0.02$ by *t*-test). This change was selective for muscular afferents; muscular/mucosal afferents' responses to stretch (Fig. 4*C*) and response threshold (32.9 ± 8.4 mN, $n = 16$, $N = 6$ in control vs. 36.1 ± 6.9 mN, $n = 21$, $N = 7$ in AHS-treated mice) were unchanged at day 11–15.

DISCUSSION

This study documents how and which classes of colorectal afferents are modulated by intraluminal hypertonicity and acidity, two outcomes of CM, suggesting a causal correlation between these chemophysical properties of luminal contents and abdominal pain. Furthermore, this study provides experimental evidence for a potential contribution of CM to the development of persistent colorectal hypersensitivity to mechanical stimulation without structural damage to the colorectum as in functional gastrointestinal disorders such as IBS.

Direct application of AHS onto the luminal side of receptive endings in vitro excited most serosal afferents and a group of MIAs in naïve colorectum, which corresponds well with previous findings in rat lumbar splanchnic nerves where 55% and 30% of colon serosal afferents were excited by hypertonic saline and HCl, respectively (26). In earlier works by Haupt et al. (17), 14% of unclassified colon afferents were found to be excited by hypertonic saline in cat inferior splanchnic nerves.

AHS also had a reversible inhibitory effect on stretch-sensitive afferent classes. The underlying mechanism(s) of the divergent effects of direct application of AHS on serosal afferents/MIAs and stretch-sensitive afferents is currently unclear. It can be postulated that multiple mechanisms are involved; for instance, release of soluble mediators, such as neuropeptides or 5-HT, may account for the AHS-induced excitation of serosal afferents and MIAs as reported in trachea and duodenum upon hypertonic stimulation (31, 34), whereas the AHS-induced inhibition of stretch-sensitive afferents may be attributed to conformational changes in neuronal membranes (shrinkage due to hypertonicity), which would reduce membrane tension and decrease the force delivered to stretch transducers.

The net result of intracolonic AHS exposure in vivo was excitatory because it increased activation of colon DRG neurons (demonstrated as increased pERK expression). Extrapolating from the in vitro effects of direct application of AHS, neurons expressing pERK in response to intracolonic AHS may be assumed to be in vivo counterparts of serosal afferents and MIAs that discharged action potentials when acutely exposed to AHS in electrophysiological recordings in vitro, which could function as proton/osomosensitive afferents, detecting chemophysical properties of luminal contents.

Assuming that intracolonic instillation of AHS inhibited stretch-sensitive afferent classes in vivo as effectively as in vitro, peripheral afferent drive encoding colorectal wall stretch would be expected to be decreased following AHS exposure (at least until stretch-sensitive afferents recover from the effects of AHS, which they do in vitro), and would thus result in an attenuation of VMRs to CRD. Unexpectedly, however, VMRs

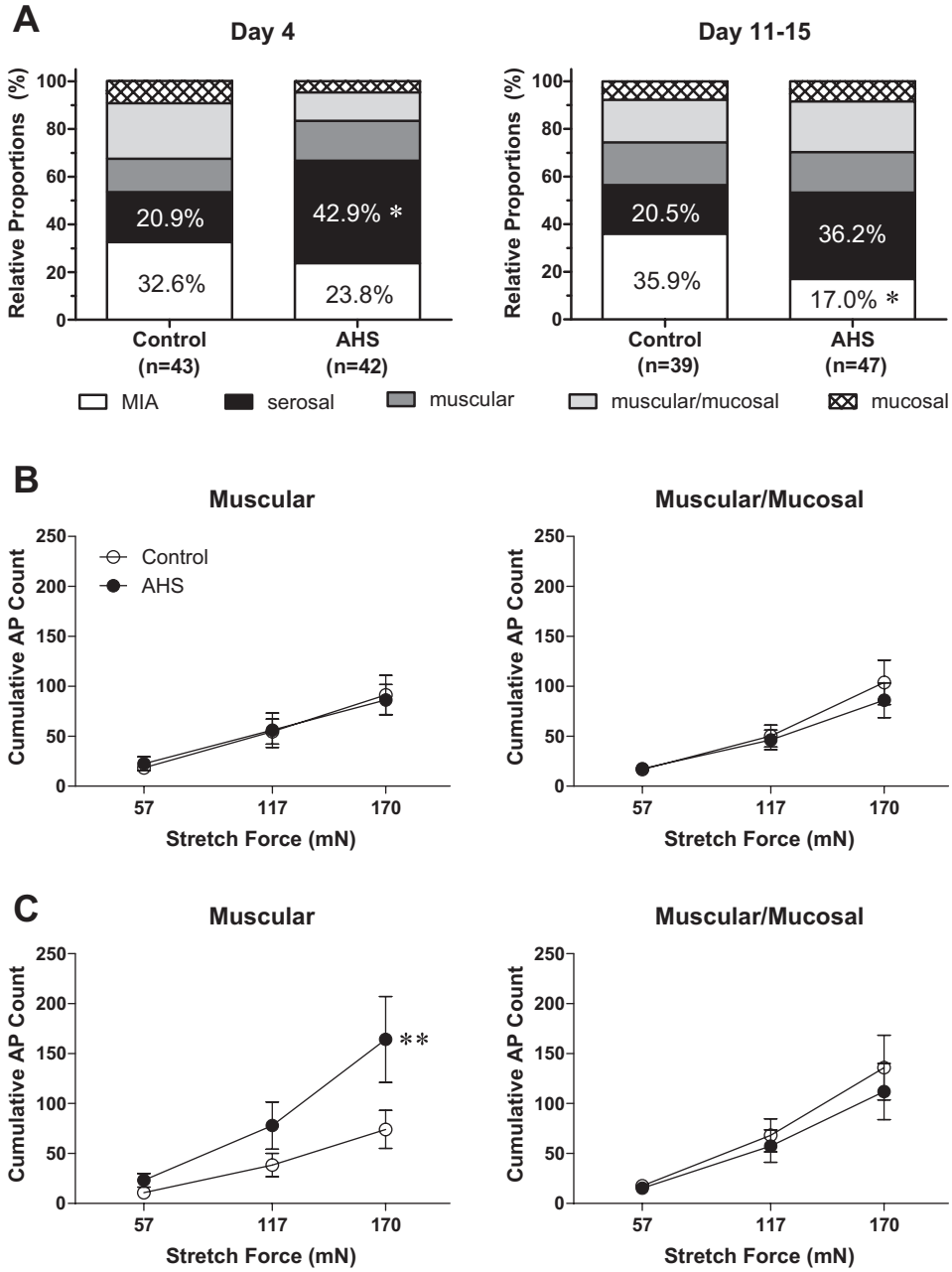

Fig. 4. Persistent changes in colorectal afferents by AHS. Mice received daily intracolonic instillation of AHS or saline for 3 consecutive days and were studied 4 and 11–15 days after the first intracolonic treatment. *A*: relative proportions of colorectal afferent classes in control and AHS-treated mice 4 (*left*) and 11–15 (*right*) days after the first intracolonic AHS treatment. $N = 4$ for each group. $^{*}P < 0.05$ vs. control by Fisher's exact test. *B*: stretch-encoding of muscular (*left*, $n = 13$, $N = 5$ in control and $n = 11$, $N = 6$ in AHS) and muscular/mucosal (*right*, $n = 19$, $N = 6$ in control and $n = 12$, $N = 4$ in AHS) afferents 4 days after the first AHS intracolonic treatment. *C*: stretch-encoding of muscular (*left*, $n = 20$, $N = 7$ in control and $n = 17$, $N = 7$ in AHS) and muscular/mucosal (*right*, $n = 16$, $N = 6$ in control and $n = 21$, $N = 7$ in AHS) afferents 11–15 days after the first AHS intracolonic treatment. $^{**}P < 0.01$ vs. control by 2-way RM ANOVA followed by Bonferroni's post hoc test.

to CRD in vivo were not affected immediately after AHS application; rather, a delayed sensitization to CRD developed. The preservation of VMRs after AHS could be hypothetically explained by *1*) central modulation of CRD-evoked peripheral inputs, *2*) recruitment of the lumbar splanchnic colorectal afferent pathway in VMRs, and/or *3*) new or augmented colorectal afferent inputs generated by CRD in other than the stretch-sensitive afferent classes studied. With any of these, the delayed sensitization to CRD could be accounted for by gradual recovery of stretch-sensitive afferents from AHS-induced inhibition.

In regard to the notion that CRD could generate new sensory inputs in afferents other than muscular and muscular/mucosal afferents following acute AHS instillation, it is noteworthy that AHS sensitized a group of MIAs to acquire mechanosensitivity to mechanical probing. Although it is not clear how these afferents that only respond to blunt probing in vitro could contribute to the VMR to CRD (unless the mechanosensitivity of sensitized MIAs was underestimated in the in vitro recording conditions used in the current study), sensitization of MIAs seems to be associated with colorectal hypersensitivity (discussed below in detail).

AHS induced a persistent colorectal hypersensitivity to CRD when applied daily for 3 days. The hypersensitivity developed and was maintained in the absence of leukocytes-involved intestinal inflammation and structural changes, suggesting similarity to functional gastrointestinal disorders such as IBS. The substrates of colorectal hypersensitivity appear to include alterations in the relative proportions and properties of colorectal afferents. Four days after the initial intracolonic instillation of

AHS, there was a significant increase in the proportion of pelvic nerve serosal afferents without any changes in the proportions of other mechanosensitive afferents. At *days 11–15*, on the other hand, the proportion of MIAs was significantly decreased together with a tendency toward an increase in the proportion of serosal afferents. These opposing changes in the proportions of serosal afferents and MIAs are intriguing especially when considering that sensitized MIAs behaved like serosal afferents when acutely and directly exposed to AHS in electrophysiological recordings. This phenotypic conversion of MIAs to serosal-like afferents after sensitization was also reported in a study where a cocktail of inflammatory mediators was applied to MIA endings (13), suggesting that MIAs may have been persistently sensitized in AHS-treated mice and characterized as serosal afferents. We previously observed similar shifts in the relative proportions of these two afferent classes in another model of persistent colorectal hypersensitivity in which structural damage to the colorectum is also absent after intracolonic zymosan treatment (14). In contrast, the relative proportions of pelvic nerve mechanosensitive afferents were reported unchanged, both in an acute inflammatory and a late recovery phase, in a model of TNBS-produced colitis in mice (18); MIAs were not included in that study. In the recovery phase, however, the mechanosensitivity of serosal afferents was reported to be increased, which is in contrast to previous findings in the zymosan model where no difference was detected in response magnitudes of serosal afferents to graded probing (14). In the present study, we did not quantify the mechanosensitivity of serosal class afferents in AHS-treated mice, which needs to be addressed in subsequent studies.

In addition to the persistent changes described above, muscular class stretch-sensitive afferents were selectively sensitized at *days 11–15*, suggesting a contribution to AHS-induced colorectal hypersensitivity. That this increase was not observed at *day 4* suggests that the underlying mechanism(s) of AHS-induced colorectal hypersensitivity is different between early (*day 4*) and late (*days 11–15*) phases. The delayed development of sensitization in muscular afferents after repeated AHS treatment suggests that a time-dependent process took place, such as a change in gene expression, synthesis of new proteins and transport of molecules to their terminal endings. It is unclear at present why such changes occurred only in muscular afferents and not also in stretch-sensitive muscular/mucosal afferents. With respect to this selective sensitizing effect on stretch-sensitive muscular afferents, intracolonic TNBS is also associated with sensitization of muscular afferents [2-wk after treatment, a time when colorectal hypersensitivy is significant (12)], whereas this afferent class is not affected in the zymosan model (14) in which muscular/mucosal afferents are persistently sensitized. These findings suggest that the two stretch-sensitive afferent classes may have distinct cellular machinery for mechanosensitivity and can be differentially modulated.

Searching for molecules that mediate afferent sensitization, in this case AHS-induced activation/sensitization of colorectal afferents, is of obvious interest for a future study. In this regard, it is particularly interesting that the majority of colorectal afferents express acid-sensing ion channel 3 (19) and transient receptor potential channels V1, V4, and A1 (6, 24) that are sensitive to protons (30) and/or hypertonicity (1, 25, 28, 33). Moreover, transgenic mice lacking these molecules exhibit decreased sensitivity to CRD (4, 6, 20, 21), suggesting a potential involvement of these molecules in the AHS-induced colorectal hypersensitivity to CRD.

In summary, we found that luminal hypertonicity and acidity, two outcomes of CM, not only acutely modulated the activities of colorectal afferents but also induced a weeks-long colorectal hypersensitivity when instilled into the colon lumen. Direct application of AHS activated a group of serosal afferents and MIAs, suggesting a role for these afferent classes in acute colorectal nociception. Daily intracolonic AHS for 3 days produced a robust colorectal hypersensitivity associated with an increase in the relative proportion of serosal afferents (and commensurate decrease in the proportion of MIAs) together with sensitization of muscular class stretch-sensitive afferents. These results suggest CM as a potential predisposing factor for the development of chronic abdominal discomfort/pain in the absence of structural damage as in IBS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.H.L. conception and design of research; J.H.L., B.F., E.S.S., and P.R.B. performed experiments; J.H.L., B.F., E.S.S., and P.R.B. analyzed data; J.H.L., B.F., E.S.S., P.R.B., and G.F.G. interpreted results of experiments; J.H.L. and B.F. prepared figures; J.H.L. drafted manuscript; J.H.L., B.F., E.S.S., and G.F.G. edited and revised manuscript; J.H.L., B.F., E.S.S., and G.F.G. approved final version of manuscript.

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