Afferent nerve sensitivity is decreased by an iNOS-dependent mechanism during indomethacin-induced inflammation in the murine jejunum in vitro

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Abstract

Evidence exists that visceral afferent sensitivity is subject to regulatory mechanisms. We hypothesized that afferent sensitivity is decreased in the small intestine during intestinal inflammation by an inducible nitric oxide synthase (iNOS)-dependent mechanism. C57BL/6 mice were injected twice with vehicle or 60 mg kg(-1) indomethacin subcutaneously to induce intestinal inflammation. Afferent sensitivity was recorded on day 3 from a 2-cm segment of jejunum in vitro by extracellular multi-unit afferent recordings from the mesenteric nerve bundle. In subgroups (n = 6), iNOS was inhibited selectively by L-N6-(1-iminoethyl)-lysine (L-NIL) given either chronically from day 1-3 (3 mg kg(-1) twice daily i.p.) or acutely into the organ bath (30 mumol L(-1)). The indomethacin-induced increase of macroscopic and microscopic scores of intestinal inflammation (both P < 0.05) were unchanged after pretreatment with L-NIL. Peak afferent firing following bradykinin (0.5 mumol L(-1)) was 55 +/- 8 impulse s(-1) during inflammation vs 97 +/- 7 impulse s(-1) in controls (P < 0.05). Normal firing rate was preserved following L-NIL pretreatment (112 +/- 16 impulse s(-1)) or acute administration of L-NIL (108 +/- 14 impulse s(-1)). A similar L-NIL dependent reduction was observed for 5-HT (250 mumol L(-1)) and mechanical ramp distension from 20 to 60 cmH(2)O (both P < 0.05). Intraluminal pressure peaks were decreased to 0.66 +/- 0.1 cmH(2)O during inflammation compared to 2.51 +/- 0.3 in controls (P < 0.01). Afferent sensitivity is decreased by an iNOS-dependent mechanism during intestinal inflammation which appears to be independent of the inflammatory response. This suggests that iNOS-dependent nitric oxide production alters afferent sensitivity during inflammation by interfering with signal transduction to afferent nerves rather than by attenuating intestinal inflammation.
Afferent nerve sensitivity is attenuated by an iNOS dependent mechanism during indomethacin-induced inflammation in the murine jejunum in vitro.

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Abstract (250 words)

**Introduction:** Evidence exists that visceral afferent sensitivity is subject to regulatory mechanisms. We hypothesized that afferent sensitivity is increased in the small intestine during intestinal inflammation by an iNOS dependent mechanism.

**Methods:** C57BL/6 mice were injected twice with vehicle or 60 mgkg⁻¹ indomethacin subcutaneously to induce intestinal inflammation. Afferent sensitivity was recorded on day 3 from a 2 cm segment of jejunum *in vitro* by extracellular multi-unit afferent recordings from the mesenteric nerve bundle. In subgroups (n=6), the inducible NO-Synthase was selectively inhibited by L-N6-(1-iminoethyl)-lysine (L-NIL) given chronically from day 1-3 (3 mg kg⁻¹ twice daily i.p.) or acutely in the organ bath (30 µM).

**Results:** Macroscopic and microscopic scores of intestinal inflammation were increased following indomethacin (both p<0.05) but unchanged during L-NIL administration. Peak afferent firing following bradykinin (0.5 µM) was 55±8 impsec⁻¹ during inflammation compared to 97±7 impsec⁻¹ in controls (p<0.05). This reduced discharge during inflammation was missing following chronic treatment with L-NIL (112±16 impsec⁻¹) or its acute administration in the organ bath (108±14 impsec⁻¹). A similar L-NIL dependent reduction observed for 5-HT (250 µM) and mechanical ramp distension from 20 to 60 cm H₂O (both p<0.05). Intraluminal pressure peaks were decreased to 0.66±0.1 cmH₂O during inflammation compared to 2.51±0.3 in controls (p<0.01).

**Conclusions:** Afferent sensitivity is decreased by an iNOS dependent mechanism during intestinal inflammation which appears to be independent of the inflammatory response. This suggests that nitric oxide synthesis by iNOS alters afferent sensitivity during inflammation by interfering with signal transduction to afferent nerves rather than by attenuating the inflammatory response.

**Keywords:** Inflammatory bowel disease, intestinal inflammation, nitric oxide, visceral sensitivity
Introduction

An abundance of evidence was accumulated in recent years supporting the hypothesis that visceral hypersensitivity is the key pathophysiological mechanisms underlying functional bowel disorders such as functional dyspepsia and irritable bowel syndrome (1). This concept implies that in patients suffering from these disorders, physiological stimuli from the gastrointestinal tract that are not consciously perceived by healthy individuals are realized by diseased patients and sensed as pain and other abdominal symptoms. Thus, these patients seem to have a lowered sensory threshold for these physiological visceral stimuli or an increased sensory gain in the afferent autonomic nervous system or both (2). As a consequence, an enormous effort was undertaken worldwide, to identify mechanisms that regulate this afferent sensitivity in order to provide targets for therapeutic pharmacological intervention.

While visceral sensory mechanisms attracted tremendous attention in the context of functional bowel disorders, regulation of sensation in the gastrointestinal tract is not limited to these disorders. Inflammatory bowel disease (IBD) is characterized by abdominal symptoms secondary to intestinal inflammation. The extent of inflammation often does not correlate well with the magnitude of the symptoms perceived by the patients (3) suggesting that regulation of sensory mechanisms may also occur during IBD. Indeed, Mayer et al. has shown in IBD patients that they have an increased threshold to visceral mechanical stimulation which was applied by balloon distension in the rectum (4). Thus, sensory mechanisms from the gastrointestinal tract appear to have the potential to be regulated in both ways. Upregulation - as seen in functional bowel disorders - may lower sensory thresholds for visceral stimuli,
while downregulation may occur in the context of intestinal inflammation as seen during IBD (5). One strategy, therefore, to explore these mechanisms is to study models of intestinal inflammation in more detail rather than focusing on models of hypersensitivity.

One elegant way to elucidate mechanisms for the regulation of afferent sensitivity in the context of irritable bowel syndrome or intestinal inflammation is to directly record from mesenteric afferent nerves in rodents. In the DSS model of colitis, Coldwell et al. investigated mechanosensitivity and sensitivity to 5-HT by direct afferent nerve recordings. They observed increased afferent sensitivity of splanchnic afferents to 5-HT and the mast cell deganulator compound 48/80 during acute intestinal inflammation (6). Furthermore, Wynn et al. observed in the TNBs-colitis model that ATP has an enhanced role in mechanosensory transduction during inflammation (7). These studies were limited to experimental models of colitis representing only one part of the gut potentially afflicted during IBD since Crohn’s disease patients frequently have inflammation in the small intestine. Although it was shown that ATP, 5-HT and other mast cell mediators are at play when afferents are sensitized during intestinal inflammation, it remained unresolved, whether these mediators generally alter the visceral sensory threshold during inflammation.

In our own work that involved a systemic LPS-induced inflammatory response, we found that nitric oxide synthesized by inducible nitric oxide synthase (iNOS, 8) seems to be one candidate which may have the potential to regulate intestinal afferent sensitivity during inflammation. This work, however, was based on a model simulating sepsis rather than intestinal inflammation, so that the prime question whether nitric oxide would also regulate
afferent sensitivity during intestinal inflammation remained unresolved.

We, therefore, chose a model of small intestinal inflammation that is established for the study of mechanisms contributing to inflammatory bowel disease (9). Our specific hypothesis was that afferent nerve discharge would be decreased during inflammation mediated via an iNOS dependent pathway independent of the extent of inflammation.
Methods

Animals

Experiments were performed with male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing approximately 20g. Animals were kept under a 12h/12 h dark/light cycle and had free access to food and water until they were sacrificed. Animal experiments were approved by the local Institutional Review Board.

Induction of intestinal inflammation

Intestinal inflammation was induced by 2 subcutaneous injections of 60 mg kg⁻¹ indomethacin at 8 a.m. on day 1 and day 2 (volume 1.5 ml kg⁻¹; ethanol 100 % as vehicle). The timing and dose of indomethacin were determined considering previous reports in rat and by preliminary pilote experiments to ensure that a moderate intestinal inflammation was induced avoiding necrotic (black) intestine and limiting maximum lethality to below 40 % of the animals. Control animals were injected with vehicle.

Surgical procedure / Assessment of inflammation

One day after the 2nd and last indomethacin/vehicle injection, animals were deeply anesthetized at 9 a. m. with isoflurane inhalation. Then, a laparotomy was performed and the small intestine exteriorized and inspected. Inflammation was quantified by a macroscopic inflammation score which included evaluation of adhesions, intestinal length, hyperemia and ulcers (range 0-8 points; Table 1a). This macroscopic score was previously published for rat by Yoshida (10) and modified in our study in order to be suitable for the evaluation of mice
"Intestinal shortening" was assessed instead of "wall thickness". The description of ulcers was limited to "none", "single" or "multiple", while the parameter "hyperemia" was amended by the category "intestinal bleeding". The appearance of the intestine was additionally photo-documented with a digital camera, so that the assessment could be double-checked later on.

Furthermore, a segment of the jejunum just distal to the ligament of Treitz was excised, fixed in 4 % normal buffered formalin and processed for histology. Microscopic assessment of inflammation in this tissue was performed by a blinded observer on slides stained with haematoxylin and eosin. Inflammation was quantified with the help of a previously published microscopic score system (Table 1b, 11).

**Afferent nerve recording in vitro**

A segment of proximal jejunum with a length of 2 cm and the mesentery attached was carefully excised before the animal was killed with an overdose of isoflurane. Multi-unit afferent nerve recordings were established in a custom-made organ bath consisting of two chambers i. e. a perfusion and a recording chamber as published previously (12). The intestinal segment was placed into the perfusion chamber of the organ bath and superfused with Kreb’s buffer which was equilibrated with O\textsubscript{2}/CO\textsubscript{2} mixture (composition of Kreb’s (mM): Na\textsuperscript{+} 143.5, K\textsuperscript{+} 5.9, Cl\textsuperscript{−} 126, Ca\textsuperscript{2+} 2.5, Mg\textsuperscript{2+} 1.2, H\textsubscript{2}PO\textsubscript{4} 1.2, SO\textsubscript{4} 1.2, HCO\textsubscript{3}\textsuperscript{−} 25, glucose 10 and sodium butyrate 1, pH 7, superfused at a rate of 10 ml min\textsuperscript{−1}, temperature 32°C). Both ends of the jejunal segment were cannulated and the lumen of the prepared segment was continuously perfused with Kreb’s from the proximal side of the jejunum (10 ml per hour),
while the distal cannula remained open to the atmosphere during the experiment unless a mechanical ramp distension was performed (see below). Perfusion and luminal distension were performed with a syringe pump (IVAC 711, IVAC Corp, San Diego, USA). The intraluminal pressure was recorded continuously through a separate channel in the proximal cannula with a pressure transducer in order to monitor intestinal motor events (Neurolog pressure amplifier NL 108, Digitimer Ltd., Welwyn Garden City, UK).

While the intestinal segment remained in the perfusion chamber, extracellular multi-unit afferent nerve recordings were established by dissection of the mesenteric nerve out of the neurovascular mesenteric bundle under a viewing microscope (Wild, M3Z, Heerburg, Switzerland). For this purpose, the mesenteric arcade was drawn through a small aperture into a separate chamber of the organ bath next to the perfusion chamber i.e. the recording chamber. The aperture was sealed with vaseline and the separate recording chamber filled with colourless heavy liquid paraffin (Sigma Chemicals, Munich, Germany) pre-warmed to 32°C for electrical insulation. Under binocular microscopic vision, the dissected mesenteric nerve was placed onto one arm of a pair of bipolar platinum recording electrodes, while a strip of connective tissue was wrapped around the other arm. The electrodes were connected to a CED single channel 1902 preamplifier/filter (Cambridge Electronic Design (CED), Cambridge, UK). The signal was amplified 10,000 times and filtered with a bandwidth of 100 Hz to 1 kHz. Signals from the pressure transducer that recorded the intraluminal intestinal pressure were transmitted to another CED single channel 1902 preamplifier/filter. The output from the 1902, together with the signals from the pressure transducer were fed into a power Micro 1401 interface system (CED) saved on the hard drive of a laptop computer, and viewed online by
running Spike 2 software (version 4.01; CED).

**Protocol for afferent nerve recording**

Once a stable baseline recording was established for 20 minutes, the afferent nerve response to serosal 5-hydroxytryptamine (5-HT, 250µM), ramp distension up to 60 cmH₂O and serosal bradykinin (BK, 0.5µM) were investigated. For the afferent response to chemical stimuli, the perfusion was stopped and 5-HT or BK was administrated in the organ bath with a pipette (added volume 250 µl from a stock solution of 5 mM for 5-HT and 10 µM for BK, volume in the perfusion chamber of the organ bath was 5 ml; the minimal volume change by mediator administration was neglected). After 2 minutes the perfusion was started again to wash out the chemical. For continuous ramp distension, the outlet cannula in the intestinal lumen was clamped, while perfusion with Kreb’s solution was continued at 10 ml h⁻¹. With this method the gut segment was distended to 60 cmH₂O in approximately 90s. Then the cannula was opened to let intraluminal pressure return to baseline. After each stimulus an interval of at least 15 minutes was allowed to have the baseline discharge recovered before the next stimulus was administered. In preliminary cross-over pilot experiments, we had previously determined that there is no interaction with the different test stimuli concerning the afferent nerve response when administered in this order. Furthermore, doses of bradykinin and 5-HT were based on preliminary dose-finding studies to ensure that submaximal doses were chosen (data not shown).
**Experimental subgroups**

Afferent nerve recordings were obtained on day 3 after the beginning of different forms of pretreatment leading to 4 subgroups of animals (each n=6):

1) Mice that were injected with indomethacin (60 mg kg\(^{-1}\) s.c.) on day 1 and 2 at 8 a.m. to induce intestinal inflammation.

2) Mice that were injected with vehicle (100 % ethanol, volume 1.5 ml kg\(^{-1}\) s.c.) on day 1 and 2 at 8 a.m. as controls for indomethacin induced inflammation.

3) Mice that were injected with indomethacin (60 mg kg\(^{-1}\) s.c.) on day 1 and 2 at 8 a.m. to induce intestinal inflammation plus *chronic* treatment with the selective iNOS inhibitor L-N6-(1-iminoethyl)-lysine (L-NIL). L-NIL was injected into the peritoneum at a dose of 3 mg kg\(^{-1}\) (total volume 0.2 ml) at 8 a.m. and 8 p.m. on day 1 and 2 as well as on day 3 at 8 a.m. (total of 5 injections). Thus, the last dose was administered 1 hour before the intestinal segment was taken out for afferent nerve recordings. The dose of L-NIL was chosen according to previous publications (13, 14).

4) Mice that were injected with indomethacin (60 mg kg\(^{-1}\) s.c.) on day 1 and 2 at 8 a.m. to induce intestinal inflammation. In this subgroup, L-NIL was dissolved in Kreb’s solution and administered at a concentration of 30 µM *acutely* in the organ bath 10 minutes prior to application of the test stimuli 5-HT, ramp distension and bradykinin. Again, this concentration of L-NIL was chosen according to a previously published report (15).

In some additional control experiments, vehicle (ethanol) pre-treated mice received either intraperitoneal L-NIL *chronically* (as described in (3)) or *acutely* in the organ bath (as described in (4)). Neither intraperitoneal nor L-NIL administration in the organ bath altered
afferent nerve discharge to 5-HT, luminal ramp distension or bradykinin in these additional control animals without intestinal inflammation (data not shown).

**Drugs**

Indomethacin, L-NIL, 5-HT and bradykinin were purchased from Sigma, Munich, Germany. Isoflurane was obtained from Abbott, Wiesbaden, Germany. L-NIL was dissolved in normal saline for i.p. injection and in Kreb’s solution for perfusion in the organ bath. 5-HT and bradykinin were dissolved in normal saline.

**Data analysis**

The baseline discharge frequency (imp sec\(^{-1}\)) was determined by averaging the afferent nerve discharge during the 2 minute recording period prior to administration of test stimuli. The afferent nerve response to chemical stimulation with 5-HT and BK was evaluated as the increase in peak impulse frequency per second above baseline discharge frequency during a 3 second period of maximum afferent firing. The response to ramp distension was evaluated by quantifying the peak impulse frequency per second over a 3 second period at 10 cm H\(_2\)O increments of intraluminal pressure until 60 cmH\(_2\)O were reached. Intestinal motility was quantified at baseline by determining the mean of the pressure peaks during spontaneous phasic changes in intraluminal pressure for a 30 second interval before test stimuli were administered. Following the chemical stimuli BK and 5-HT, intestinal pressure events were quantified as the peak luminal pressure above baseline within 2 minutes after administration of the stimulus. Data are presented as mean ± SEM and were compared by one-way ANOVA and post-hoc Bonferroni correction.
Results

Intestinal inflammation

Following indomethacin pretreatment, animals developed acute intestinal inflammation in the whole small intestine which was characterized by obvious intestinal dilation, marked reduction in small intestinal length and adhesions among small intestinal loops. Furthermore, intestinal inflammation was typically accompanied by hyperemia, ulcers of the gut wall and hemorrhage into the lumen. Elevated scores were observed with the macroscopic scoring system (see Table 1a) in animals pretreated with indomethacin compared to controls. After chronic pretreatment with the selective iNOS inhibitor L-NIL, intestinal inflammation was macroscopically not different from unpretreated animals. Figure 1A and B shows representative photographs and the macroscopic scores are given in Table 2.

Intestinal inflammation was also assessed histologically in Haematoxylin & Eosin stains. Histology of inflamed intestine showed that the villi lost their normal upright fingerlike appearance. Villi were typically bent or tilted and further characterized by hyperemia and edema. The normal cellular architecture of the villi was destroyed. In some the erosion of the mucosal side was serious and many of the villi were absent (Figure 1C and D). After chronic pretreatment with the selective iNOS inhibitor L-NIL, intestinal inflammation was histologically not different from unpretreated animals. Details of the evaluation with a previously published score system (see Table 1b) is given in Table 2.

Baseline afferent nerve discharge and motility

Once the preparation for multi-unit afferent nerve recordings was established, continuous
afferent firing was present at baseline which consisted of spikes with different amplitudes and waveforms. Baseline discharge was not different among the various subgroups (20±3.2 imp sec⁻¹ in vehicle controls; 13±3.2 imp sec⁻¹ after indomethacin pretreatment; 18±2.9 imp sec⁻¹ after indomethacin pretreatment plus chronic L-NIL; 19±3.4 imp sec⁻¹ after indomethacin pretreatment plus acute L-NIL in the organ bath; all n=6, differences not significant).

Spontaneous intestinal motor activity was observed in segments from control mice which was characterized by regular phasic contractions. This contractile pattern was not present in indomethacin pre-treated animals (Figure 2). The mean peak intraluminal pressure during two minutes recording period before administration was decreased from 2.51±0.3 cmH₂O in controls to 0.66±0.1 cmH₂O in indomethacin pre-treated animals (p<0.05; n=6). In animals with inflamed small intestine secondary to indomethacin, additional chronic L-NIL pre-treatment i. p. or acute L-NIL administration in the organ bath was followed by mean peak pressures of 0.80±0.1 cmH₂O and 1.56±0.3 cmH₂O which were also different from control animals (both p<0.05; n=6).

Afferent nerve discharge to chemical and mechanical stimulation

Afferent nerve discharge increased promptly following 5-HT administration into the organ bath at a concentration of 250 µM (Figure 2). The raw nerve trace revealed that this increased was generated by discharge of additional units that were recruited during the response and by spontaneously active fibers that increased their firing frequency. In vehicle controls maximum firing was 65±7.5 imp sec⁻¹ above baseline, which was reduced to 32±3.3 imp sec⁻¹ in animals with indomethacin induced inflammation (P<0.05). This reduction in afferent firing to 5-HT
was not seen in animals with intestinal inflammation following chronic intraperitoneal pre-
treatment with L-NIL or after acute L-NIL administration in the organ bath (Figure 2D). As
regards intestinal motility, 5-HT virtually abolished the regular motility pattern observed at
baseline in control animals (Figure 2). Peak pressures were 2.62±0.4 cmH\textsubscript{2}O at baseline and
1.59±0.2 cmH\textsubscript{2}O after 5-HT (p<0.05). Motor events were unchanged before and after 5-HT
exposure in animals with inflamed small intestine (peak pressure 0.97±0.1 cmH\textsubscript{2}O before and
1.08±0.1 cmH\textsubscript{2}O after 5-HT, n.s.). This situation was the same for chronic or acute L-NIL
administration during intestinal inflammation (chronic L-NIL: peak pressure 1.16±0.18
cmH\textsubscript{2}O before and 1.34±0.11 cmH\textsubscript{2}O after 5-HT, n.s.; acute L-NIL: peak pressure 1.48±0.3
cmH\textsubscript{2}O before and 1.52±0.2 cmH\textsubscript{2}O after 5-HT, n.s.).

During mechanical stimulation by ramp distension a pressure dependent increase in afferent
nerve discharge occurred in all experimental subgroups (Figure 3). When compared to
controls, mechanical sensitivity was reduced during intestinal inflammation for luminal
distension pressures that were exceeding 20 cmH\textsubscript{2}O (p<0.05, Figure 3D). At the maximum
pressure of 60 cmH\textsubscript{2}O, afferent firing was 38±3.0 imp sec\textsuperscript{-1} in inflamed intestinal segments
and 95±5.9 imp sec\textsuperscript{-1} in controls (p<0.05). Peak afferent nerve discharge was unchanged
during the complete mechanical ramp distension following chronic adnminstration of the
selective iNOS inhibitor L-NIL by repeated intraperitoneal injections or acute administration
in the organ bath when compared to controls (Figure 3D).

Bradykinin (BK) superfusion in the organ bath was followed by an obvious and robust
increase in afferent nerve discharge (Figure 4). This increase consisted of new recruitment of
previously silent units and increase firing frequency of spontaneously active fibers. While peak afferent firing was 97±7.0 imp sec\(^{-1}\) in intestinal segments from vehicle control animals, it declined to 55±7.9 imp sec\(^{-1}\) in inflamed segments after indomethacin pre-treatment (p<0.05, Figure 4D). Following pharmacological inhibition of iNOS during inflammation with the selective agent L-NIL administered either chronically (i.p.) or acutely in the organ bath, peak afferent firing was 112±16.0 imp sec\(^{-1}\) and 108±13.7 imp sec\(^{-1}\) which was not different from the response in uninflamed intestinal segments from vehicle control animals.

In uninflamed intestinal segments from vehicle control animals, bradykinin was followed by an inhibition of regular phasic contractions (Figure 4) This was quantified by peak intraluminal pressures that were 1.68 ± 0.3 cmH\(_2\)O before and 2.1 ± 0.4cmH\(_2\)O after bradykinin superfusion (n.s.). The motor response to bradykinin was different in inflamed intestinal segments from indomethacin pre-treated animals. Here, phasic intestinal contractions were virtually absent before bradykinin administration but a single tonic rise in intraluminal pressure was observed which occurred obviously unrelated to the peak increase in afferent firing. This tonic pressure peak was 6.7±2.4 cmH\(_2\)O in inflamed segments compared to the intraluminal pressure of 0.9±0.1 cmH\(_2\)O at baseline (p < 0.001). The tonic increase in intraluminal pressure following bradykinin compared to baseline was also observed in inflamed intestinal segments following chronic L-NIL pre-treatment i.p. which was 1.1±0.1 cmH\(_2\)O before and 5.4±0.4 cmH\(_2\)O (p<0.001), and following acute administration of L-NIL into the organ bath 1.4 ± 0.2 cmH\(_2\)O before and 8.9 ± 1.0cm H\(_2\)O after acute L-NIL administration (p<0.001).
Discussion

In the present study, alterations in afferent sensitivity were investigated at the level of the mesenteric nerve during small intestinal inflammation induced by indomethacin in mice which was previously described as a model for inflammatory bowel disease (9, 16). Peak afferent firing to mechanical ramp distension of the intestinal loop and to chemical stimulation by superfusion with bradykinin or 5-HT in the organ bath *in vitro* was attenuated during inflammation compared to control animals. Hyporesponsiveness of the afferent nerve during intestinal inflammation was reversed to control levels following pretreatment with the selective iNOS inhibitor L-NIL. This was observed for both chronic L-NIL administration with repeated intraperitoneal injections during the induction phase of inflammation and acute L-NIL administration in the organ bath 10 minutes prior to chemical or mechanical stimulation. Interestingly, chronic L-NIL pretreatment did not attenuate the extent of intestinal inflammation following indomethacin which was quantified by a macroscopic and microscopic scoring system. Spontaneous intestinal motility was virtually absent in the jejunal segment during inflammation which was also uninfluenced by previous L-NIL pretreatment.

Inflammation develops in the intestine following indomethacin administration since the mucosa is depleted of prostaglandins (17, 18). Subsequently, ulcers and increased intestinal permeability develop that allows luminal contents such as bacterial wall components (e.g. lipopolysaccharides) to invade the intestinal wall with a subsequent development of inflammation (16, 19, 20, 21). These trigger an acute phase response including the release of proinflammatory cytokines which subsequently entertain the intestinal inflammatory response.
that was described in this study and also characterized by others (16, 21). It is of note that although indomethacin induced intestinal inflammation was described as a model for inflammatory bowel disease (9), it represents this disease entity only to a limited extent. Limiting factors are in particular that the inflammation develops subsequent to depletion of prostaglandin, while increased prostaglandin production is a feature of inflammatory bowel disease in humans (22, 23), and that inflammation was studied after several days so that it may lack typical features of chronic inflammation. Nevertheless, this model was chosen since we aimed to study afferent nerve sensitivity in the small intestine and relatively few models with intestinal inflammation are available to induce inflammation in this part of the gut (9). Furthermore, our focus was to study modulation of afferent sensitivity during intestinal inflammation mainly to learn more about modulatory mechanisms of afferent sensitivity in general rather than to investigate a specific aspect of inflammatory bowel disease.

One of the most intriguing findings in the present study is that afferent sensitivity was reduced during small intestinal inflammation which was secondary to indomethacin pretreatment. This is in contrast to other reports on visceral sensitivity in different experimental models of intestinal inflammation (6, 7, 24). One difference is that previous reports investigated mostly the intrinsic and extrinsic sensory innervation during colitis, while our study was on small intestinal inflammation. It appears rather unlikely, however, that mechanisms of afferent sensitivity during inflammation are fundamentally different in the small and large intestine, so that the observed hyposensitivity is more likely to be secondary to the specific alterations in intestinal homeostasis following indomethacin administration rather than regional differences. Simple destruction of afferent nerve terminals can be ruled out as an explanation since the
reduced sensitivity was pharmacologically reversible with the iNOS inhibitor L-NIL. Non-steroidal antiinflammatory drugs entail depletion of prostaglandins in the intestinal mucosa (17, 18). As prostanoids have the potential to sensitize intestinal afferents (25), it appears to be the most likely explanation that their general depletion led to the overall reduced visceral sensitivity during indomethacin induced inflammation in this study. Alternatively, the developing intestinal inflammation may have been accompanied by a downregulation of afferent sensitivity via a different mechanism. Considering our previous work with a systemic model of LPS induced inflammation, intestinal inflammation may have induced the synthesis of iNOS with the subsequent release of nitric oxide as a potential mechanism to reduce afferent sensitivity to other stimuli as shown in the systemic LPS model (8). Thus, this mechanism was further investigated.

We found that attenuated afferent sensitivity following intestinal inflammation was reestablished to control levels following chronic administration of L-NIL, while inflammation developed. One possible explanation, why afferent sensitivity was reestablished to control levels is that inhibition of iNOS by L-NIL simply attenuated the inflammatory response brought on by indomethacin. Indeed, iNOS induction was described in several reports during indomethacin induced intestinal inflammation as a potential consequence of the increase in intestinal permeability (18, 20) that exposes the gut wall to bacterial cell wall components such as LPS. Several reports suggest that inhibition of iNOS by L-NIL improves the inflammatory response (26, 27). This proinflammatory action of nitric oxide synthesized by iNOS was questioned by other studies suggesting that nitric oxide from iNOS may also have protective effects e.g. in septic shock or gastric ulcer disease (28, 29). Consequently, it is not
clear from previous work whether chronic L-NIL treatment would have a beneficial effect on indomethacin induced intestinal inflammation. We, therefore, quantified intestinal inflammation by two scoring systems and found that L-NIL did not alter the inflammatory response. Thus, reduced intestinal inflammation is unlikely to explain the attenuated afferent sensitivity following L-NIL pretreatment in animals with intestinal inflammation. Nitric oxide synthesized by iNOS rather seems to have the potential to downregulate afferent sensitivity during intestinal inflammation without attenuation of the inflammatory response. A potential pitfall of assessing inflammation by score systems is that specific aspects of the inflammatory response such as altered infiltration with different subset of inflammatory cells etc. may have been missed, rendering the above mentioned interpretation questionable. This interpretation, however, is nevertheless further supported by the observation that acute administration in the organ bath 10 minutes prior to recording afferent sensitivity had the same effect i.e. a reconstitution of afferent sensitivity to the level of uninfamed intestine when compared to intestinal inflammation. It is extremely unlikely that administration of L-NIL 10 minutes prior to recordings affected the inflammatory response in the intestine that was going on for more than 48 hours at that time. In addition to a potentially altered inflammatory response, changes in intestinal motor events to 5-HT and BK following L-NIL were ruled out by luminal pressure recordings as they may indirectly affected afferent sensitivity by stimulation of mechanosensitive afferents. Both experimental conditions together, i.e. chronic and acute L-NIL treatment, therefore, support the interpretation that an iNOS dependent downregulation of afferent sensitivity occurred during intestinal inflammation.
The obvious question arising from these observations is by which mechanism nitric oxide downregulates afferent sensitivity. 5-HT and BK have different mechanisms of sensitizing intestinal afferents i.e. ligand-gated ion channels for 5-HT (5-HT3 receptor, 30) and G-coupled proteins for BK (31). Furthermore, luminal ramp distension sensitizes an array of different mechanosensitive afferent of different origin which are low-threshold, high-threshold and wide-dynamic range mechanosensitive afferents (32). Since afferent firing to all these stimuli was attenuated by intestinal inflammation and reversed by L-NIL pretreatment, it appears unlikely that nitric oxide synthesized from iNOS affected these different mechanisms directly, although it has a potential to alter the G-protein mechanism as well as to alter the conductance of ion channels by S-nitrosylation (33, 34). It rather seems that nitric oxide attenuates overall afferent sensitivity and this would imply a common underlying mechanism. Indeed, nitric oxide was shown to have an inhibitory action on enteric neurons, visceral and somatic afferents signaling through increased production of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) (35, 36, 37) which we suggest to be the most likely mechanism to underly the observed attenuated afferent sensitivity during intestinal inflammation in our study.

We conclude that nitric oxide synthesized by iNOS during indomethacin induced intestinal inflammation may attenuate signal transduction to extrinsic sensory nerves without altering the inflammatory response. Nitric oxide, therefore, may represent a mechanism that allows the adaptation of visceral sensitivity to continued intestinal inflammation. Whether this mechanisms explains reduced visceral sensitivity in patients suffering from inflammatory bowel disease remains to be elucidated by further pharmacological studies.
References


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### Table 1a Score system for macroscopic inflammation (modified after reference D29)

<table>
<thead>
<tr>
<th>Adhesions (serosa and mesentery)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Minimal</td>
<td>1</td>
</tr>
<tr>
<td>Involving several loops</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intestinal shortening</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Recognized</td>
<td>1</td>
</tr>
<tr>
<td>Severe</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hyperemia</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Recognized</td>
<td>1</td>
</tr>
<tr>
<td>Luminal bleeding</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ulcers</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Single Ulcer</td>
<td>1</td>
</tr>
<tr>
<td>Multiple</td>
<td>2</td>
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</table>

### Table 1b Score system for histological assessment of inflammation (see also reference D30)

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal morphology</td>
<td>0</td>
</tr>
<tr>
<td>Loss of goblet cells</td>
<td>1</td>
</tr>
<tr>
<td>Loss of goblet cells in large areas</td>
<td>2</td>
</tr>
<tr>
<td>Loss of crypts</td>
<td>3</td>
</tr>
<tr>
<td>Loss of crypts in large areas</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infiltration</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No infiltrates</td>
<td>0</td>
</tr>
<tr>
<td>Infiltrate around crypt basis</td>
<td>1</td>
</tr>
<tr>
<td>Infiltrate reaching to <em>L. muscularis mucosae</em></td>
<td>2</td>
</tr>
<tr>
<td>Extensive infiltration reaching the</td>
<td>3</td>
</tr>
<tr>
<td><em>L. muscularis mucosae</em> and thickening of mucosa with severe oedema</td>
<td></td>
</tr>
<tr>
<td>Infiltration of the <em>L. submucosa</em></td>
<td>4</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>experimental group</th>
<th>macroscopic score</th>
<th>histology score</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle control (n=5)</td>
<td>0.2 ± 0.20</td>
<td>1.6 ± 0.60</td>
</tr>
<tr>
<td>vehicle plus L-NIL (n=3)</td>
<td>0.33 ± 0.33</td>
<td>2.0 ± 0.58</td>
</tr>
<tr>
<td>inflammation (n=5)</td>
<td>4.8 ± 0.74*</td>
<td>4.6 ± 0.51*</td>
</tr>
<tr>
<td>inflammation plus L-NIL treatment (n=4)</td>
<td>4.0 ± 0.71*</td>
<td>4.0 ± 1.08*</td>
</tr>
</tbody>
</table>

Indomethacin induced intestinal inflammation was assessed by macroscopic and microscopic scoring systems in separate experiments (D29, D30). To evaluate a potential effect of the selective iNOS inhibitor L-NIL on intestinal inflammation, it was administered *chronically* by repeated intraperitoneal injections (3 mg kg⁻¹). For details see methods. Note that L-NIL administration did not alter the intestinal tissue neither in the uninflamed (vehicle plus L-NIL versus vehicle control) nor in the inflamed condition (inflammation versus inflammation plus L-NIL treatment). Both subgroups with inflammation were different compared to vehicle control (*p<0.05*).
Photographs illustrating the macroscopic appearance of control (A) and inflamed (B) intestine after indomethacin treatment. The lower two images show representative histology from control (C) and indomethacin treated (D) small intestine (hematoxylin & eosin stains). Note the moderate macroscopic inflammation characterized by edema and shortening of the intestine, while histological examination reveals substantial alterations in the villous architecture and integrity (scale bar is **3 **µm).
Representative recordings of afferent nerve discharge to 5-HT (250µM) given in the organ bath. A: recording from a vehicle control animal without intestinal inflammation. B: response to 5-HT in an animal with indomethacin induced intestinal inflammation. C: Intestinal inflammation plus chronic L-NIL pretreatment. The upper traces showed the raw nerve recording before and after 5-HT administration, while middle traces display sequential rate histograms of mesenteric afferent nerve discharge frequency in 3 seconds bin-size as used for analysis (?). The lower traces give the luminal pressure in the jejunum. Panel D summarizes the afferent nerve discharge minus baseline per second in each experimental group including recordings from segments of animals with intestinal inflammation that received acute L-NIL administration in the organ bath (30 µM). Data on the intestinal motor response is given in the text. Note that both chronic i.e. repeated i.p. treatment with the selective iNOS inhibitor L-NIL (3 mg kg⁻¹) and its acute administration in the organ bath reversed the reduced afferent nerve discharge during intestinal inflammation when compared to vehicle controls (all n=6, mean±SEM; p<0.05 versus control).
Figure 3

A: recording from a vehicle control animal without intestinal inflammation. B: response in an animal with indomethacin induced intestinal inflammation. C: Intestinal inflammation plus chronic L-NIL pretreatment. The upper traces showed the raw nerve recording, while middle traces display sequential rate histograms of mesenteric afferent nerve discharge frequency in 3 seconds bins (as used for analysis). The lower traces give the luminal pressure in the jejunum. Panel D summarizes the afferent nerve discharge minus baseline per second in each experimental group including recordings from segments of animals with intestinal inflammation that received acute L-NIL administration in the organ bath (30 µM). Note that both chronic i.e. repeated i.p. treatment with the selective iNOS inhibitor L-NIL (3 mg kg⁻¹) and its acute administration in the organ bath reversed the reduced afferent nerve discharge during intestinal inflammation when compared to vehicle controls (all n=6, mean±SEM; p<0.05 versus control).
Representative recordings of afferent nerve discharge to BK (0.5 µM) given in the organ bath. A: recording from a vehicle control animal without intestinal inflammation. B: response in an animal with indomethacin induced intestinal inflammation. C: Intestinal inflammation plus chronic L-NIL pretreatment. The upper traces showed the raw nerve recording before and after BK administration, while middle traces display sequential rate histograms of mesenteric afferent nerve discharge frequency in 3 seconds bin-size as used for analysis (?). The lower traces give the luminal pressure in the jejunum. Panel D summarizes the afferent nerve discharge minus baseline per second in each experimental group including recordings from segments of animals with intestinal inflammation that received acute L-NIL administration in the organ bath (30 µM). Data on the intestinal motor response is given in the text. Note that both chronic i.e. repeated i.p. treatment with the selective iNOS inhibitor L-NIL (3 mg kg⁻¹) and its acute administration in the organ bath reversed the reduced afferent nerve discharge during intestinal inflammation when compared to vehicle controls (all n=6, mean±SEM; p<0.05 versus control).