Arachidonic acid metabolites follow the preferential course of cyclooxygenase pathway for the basal tone in the internal anal sphincter

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de Godoy MA, Rattan N, Rattan S. Arachidonic acid metabolites follow the preferential course of cyclooxygenase pathway for the basal tone in the internal anal sphincter. Am J Physiol Gastrointest Liver Physiol 296: G727–G734, 2009. First published February 12, 2009; doi:10.1152/ajpgi.90707.2008.—Present studies determined the roles of the cyclooxygenase (COX) versus the lipoxygenase (LO) pathways in the metabolic pathway of arachidonic acid (AA) in the internal anal sphincter (IAS) tone. Studies were performed in the rat IAS versus the nontonic rectal smooth muscle (RSM). Indomethacin, the dual COX inhibitor, but not nordihydroguaiaretic acid (NDGA), the LO inhibitor, produced a precipitous decrease in the IAS tone. However, when added in the background of indomethacin, NDGA caused significant reversal of the IAS tone. These inhibitors had no significant effect on the RSM. To follow the significance of COX versus LO pathways, we examined the effects of AA and its metabolites. In the IAS, AA caused an increase in the IAS tone (E_max = 38.8 ± 3.0% and pEC_50 = 3.4 ± 0.1). In the RSM, AA was significantly less efficacious and potent (E_max = 11.3 ± 3.5% and pEC_50 = 2.2 ± 0.3). The AA metabolites (via COXs) PGF_2α, and U-46619 (a stable analog of thromboxane A_2) produced increases in the IAS tone and force in the RSM. Conversely, AA metabolites (via 5-LO) lipoxin A_4, 5-HETE, and leukotriene C_4 decreased the IAS tone. Finally, the contractile effects of AA in the IAS were selectively attenuated by the COX-1 but not the COX-2 inhibitor. Collectively, the specific effects of AA and the COX inhibitor, the Western blot and RT-PCR analyses showing specifically higher levels of COX-1, suggest a preferential role of the COX (specifically COX-1) pathway versus the LO in the regulation of the IAS tone.

smooth muscle tone; thromboxanes; prostaglandin F_2α

the internal anal sphincter (IAS), an inner ring of smooth muscle in the anal canal, exhibits spontaneous tone that undergoes relaxation in response to the rectoanal inhibitory reflex (21). The basal tone in the IAS is one of the critical components responsible for the rectoanal continence. The IAS dysfunction, on the other hand, may lead to rectoanal incontinence and a number of other rectoanal motility disorders (1, 2, 21). Despite the critical role of the basal IAS tone in the rectoanal continence, the molecular mechanisms regulating the tone are not exactly understood. Previous studies from our laboratory show that the IAS tone is partially (~25%) regulated by a local renin-angiotensin system (RAS) both in vivo (14) and in vitro (11, 13) settings. The present studies are designed to determine the nature of extracellular signal that may provide a trigger for the remaining portion of the IAS tone.

Recent studies in the IAS (15) in agreement with those in the human and cat lower esophageal sphincter (LES) (5, 6) show that group I secreted phospholipase A_2 (sPLA_2) plays an important role in the basal tone. The LES studies show that sPLA_2 activity results in the release of arachidonic acid (AA) from the smooth muscle cell membrane phospholipids, which leads to contraction of the smooth muscle.

AA is metabolized by cyclooxygenases (COX-1 and COX-2) (20) or by 5-lipoxygenase (5-LO) (16), resulting in the formation of physiologically active metabolites (4, 17, 22, 26). Once integrated within the smooth muscle system, AA may produce different leukotrienes, lipoxins and 5-HETE (primarily via 5-LO), and thromboxanes or prostaglandins (primarily via COXs). These end products may produce variable effects in different smooth muscle preparations. The final net effect of AA, therefore, depends upon the relative importance of either the COX or 5-LO pathway and the specific effects of their end products. The effect and the role of AA metabolites in the IAS smooth muscle may provide important information on the role of COX versus 5-LO, along with their end products, in the IAS tone. Presently, such information in the IAS is not available.

In the present studies, we evaluated the effects of AA, AA-metabolizing enzymes (COX and 5-LO), and AA metabolites in the basal tone of the IAS. The data provide evidence for the distinct roles of and metabolizing enzymes in modulating the IAS basal tone. In addition, the present data show that the COX (excitatory) and 5-LO (inhibitory) pathways play opposite roles in the regulation of the IAS basal tone. Data also show that inhibition of 5-LO diverts the AA metabolic traffic toward the COX pathway.

MATERIAL AND METHODS

Tissue preparation. Male Sprague-Dawley rats (300–350 g) were euthanized by decapitation, and IAS and RSM smooth muscle strips were prepared as described before (10, 11). Briefly, using sharp dissection, the IAS and RSM strips exclusively from the circular smooth muscle layer of the muscularis propria (~0.5 mm × 7 mm) were prepared in the oxygenated Krebs’ physiological solution (KPS). The composition of KPS was as follows (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl_2, 1.16 MgSO_4, 1.01 NaH_2PO_4, 25 NaHCO_3, and 11.10 glucose. The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and was in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Measurement of isometric tension. The smooth muscle strips were transferred to 2-mL muscle baths containing oxygenated KPS at 37°C. One end of the strip was anchored at the bottom of the muscle bath

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while the other end was connected to a force transducer (model FT03; Grass Instruments, Quincy, MA). Isometric tension was measured by the PowerLab/8SP data acquisition system (ADInstruments) and recorded with Chart 4.1.2 (ADInstruments). Each smooth muscle strip was initially stretched to a tension of 0.7 g followed by 90 min of equilibration. During this period, the smooth muscle strips were replenished with fresh KPS every 20 min. The IAS smooth muscle strips were defined by their development of spontaneous tone and relaxation in response to electrical field stimulation (10 Hz, 20 V, 0.5-ms pulse duration, and 4 s of train duration). The rectal smooth muscles (RSM), on the other hand, were characterized by the lack of significant spontaneous tone and by the phasic activity.

**Drug responses.** We examined the effects of AA (0.1–300 μM) before and after pretreatment with COX inhibitor indomethacin and 5-LO inhibitor nordihydroguaiaretic acid (NDGA). For these studies, we used lower concentrations (1 μM) of these inhibitors that cause minimal decrease in the IAS tone. We also tested the effects of indomethacin and NDGA (0.1–300 μM) in basal IAS tone. Additionally, we tested the effects of 5-LO products leukotrienes B4, C4, and D4 (LTB4, LTC4, LTD4, respectively), lipoxin (LX)A4, and 5-HETE (1–300 nM for all) and the effects of COX products PGF2α, and thromboxane (Tx)A2 analog (1 nM to 100 μM). [Because of the unstable nature of TxA2 in solution, we used the synthetic stable analog of TxA2, U-46619 (9).] To determine the specific isoform of COX in the AA-increased IAS tone, some experiments were performed to determine the effects of SC-560 (COX-1 inhibitor) and rofecoxib (COX-2 inhibitor), both 0.1 and 0.3 μM, on the effects of AA. Relaxant and contractile responses in the IAS were measured and normalized by the maximal increase in the IAS tone by 100 μM bethanechol and by the maximum decrease by 50 mM EDTA, respectively, as previously described (3, 13, 18).

**Western blot analysis.** Western blot studies were performed to determine the relative expression of 5-LO, COX-1, and COX-2 in the IAS versus the RSM. The IAS and RSM tissues were isolated as described in Tissue preparation and subjected to homogenization, protein extraction, and concentration determination by the method of Lowry et al. (19). Different protein groups were then separated according to their molecular weights by gel electrophoresis and transferred onto a nitrocellulose membrane (NCM) at 4°C.

The NCM was then incubated with the specific primary antibodies (goat anti-5-LO, rabbit anti-COX-1, and goat anti-COX-2; all at 1:10,000) for 2 h at RT. After NCMS were washed with Tris-buffered saline-Tween, they were incubated with horseradish peroxidase-labeled secondary antibody (1:10,000) for 1 h at RT. The corresponding bands were visualized with enhanced chemiluminescence substrate using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and Hyperfilm MP (Amersham Bioscience).

NCMs were then stripped of antibodies using the Restore Western Blot Stripping Buffer (Pierce) for 5 min at RT and then reprobed with α-actin using the specific primary (mouse IgG 1:10,000 for α-actin) and secondary (1:10,000) antibodies. Bands corresponding to different proteins were scanned (SnapSacn.310; Agfa, Ridgefield Park, NJ), and the integrated optical density (IOD) was determined using Image-Pro Plus 4.0. The relative densities were calculated by normalizing the IOD of each blot with that of α-actin.

**RT-PCR.** Total RNAs from the IAS and RSM were isolated and purified by the acid guanidine-phenol-chloroform method (8) and the acid guanidine-phenol-chloroform method (8) and quantified by the measurement of absorbance at 260 nm on a spectrophotometer. Total RNA (2.0 μg) was subjected to first-strand cDNA synthesis using oligo(dT) primers (Promega, Madison, WI) and Omniscript RT Kit (Quiagen, Germantown, MD) in a final volume of 20 μl at 42°C for 60 min. PCR primers specific for 5-LO, COX-1, COX-2, and β-actin cDNA were designed as shown in Table 1. PCR was performed in a Promega 2× Master Mix (Promega) in a final volume of 25 μl, using a Perkin-Elmer Thermal Cycler (PerkinElmer).

![Table 1. Primers used in RT-PCRs for amplification of mRNA encoding COX-1, COX-2, 5-LO, and β-actin](https://www.ajpgi.org/content/296/4/1766/F1.large.jpg)

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This suggests that attenuation of the 5-LO inhibitory pathway in the IAS diverts the AA metabolic pathway in the direction of COX excitatory pathway. Interestingly, in the RSM, such augmentation of AA responses was not observed ($P < 0.05; \text{n} = 4$; Fig. 1B).

**Effects of indomethacin and NDGA on the IAS basal tone versus RSM force.** Indomethacin and NDGA are well known inhibitors of AA metabolism by the COXs and the 5-LO enzymes, respectively. Indomethacin concentration-dependently reduced the IAS basal tone with $I_{max} = 71.5 \pm 5.2\%$ and $pIC_{50}$ of 4.7 $\pm$ 0.3 ($n = 9$). On the other hand, NDGA and NaHCO$_3$ (the aqueous solvent for both indomethacin and NDGA) did not produce any significant effect ($P > 0.05; \text{n} = 4$; Fig. 2). In contrast with the IAS, in the RSM, neither indomethacin nor NDGA produced any significant effect. This lack of effect of the inhibitors may have been because of the absence of any significant spontaneous tone in the RSM. To rule this out, experiments were repeated where the RSM force was elevated by 100 $\mu$M bethanechol. In these experimental settings, neither indomethacin nor NDGA had any significant effect on the elevated force of the RSM (data not shown).

**Time course of indomethacin and NDGA effects on IAS basal tone.** We evaluated the effect of indomethacin and NDGA (both 100 $\mu$M) on the basal tone of the IAS over 12 minutes. Data show significant (*$P < 0.05$; $\text{n} = 4$) sustained decrease in the IAS tone with indomethacin alone, whereas NDGA has no significant effect ($P > 0.05; \text{n} = 4$). NDGA administered 5 min following pretreatment with indomethacin causes significant reversal of the suppressed basal tone (**$P < 0.05$; $\text{n} = 4$).
different time intervals. Indomethacin produced maximal decrease in the IAS tone within 5 min of administration, which was maintained for at least 20 to 40 min (n = 4). On the contrary, in the basal state NDGA produced no significant effect (P > 0.05; n = 4). However, when NDGA was administered following the peak effect of indomethacin, NDGA caused an immediate and significant increase in the IAS tone (n = 4; Fig. 3). These results suggest that a part of the initial inhibitory effect by indomethacin in the IAS may be via the inhibitory 5-LO pathway. In addition, the data suggest that because of the dominant excitatory effect of COX, the functional expression of 5-LO inhibitory pathway was only discernible following complete COX inhibition.

Effects of COX and 5-LO metabolites on IAS basal tone. We evaluated the effects of the AA metabolites produced via COXs (PGF$_{2\alpha}$ and TxA$_2$) and 5-LO (LTB$_4$, LTC$_4$, LTD$_4$, leukotrienes B$_4$, C$_4$, and D$_4$, respectively; LXA$_4$, lipoxin A$_4$; NSE, no significant effect; NP, not possible to calculate) of COX, and LTB$_4$, LTD$_4$, leukotrienes B$_4$, C$_4$, and D$_4$, respectively; LXA$_4$, lipoxin A$_4$; NSE, no significant effect; NP, not possible to calculate) of COX, and 5-LO pathway. Because of the unstable nature of TxA$_2$, we examined the effect of U-46619, which is a stable analog of TxA$_2$. U-46619 and PGF$_{2\alpha}$ produced a prominent, significant, and concentration-dependent increase in the IAS tone (n = 4; Fig. 4A). On the other hand, LTB$_4$ and LTD$_4$ produced significantly less efficacious and potent increases in the IAS tone. A similar pattern of the effect of COX pathway metabolites was observed in the RSM; however, these agents were significantly more efficacious and potent in the IAS versus the RSM (n = 4; Fig. 4, A and B, and Table 2).

Interestingly, the AA metabolites released via the 5-LO (especially LXA$_4$, 5-HETE, and LTC$_4$) pathway, rather than in-
crease, produced significant and concentration-dependent decrease in the basal tone of the IAS (n = 4; Fig. 5). Because of the absence of significant levels of basal tone in the RSM, the effects of AA metabolites that produce IAS relaxation were not examined. The values for E_max and pEC_50 are summarized in Table 2.

Protein expression of COX-1, COX-2, and 5-LO using Western blot analyses. We evaluated the presence of COX-1, COX-2, and 5-LO in protein extracts from IAS and RSM. Although both tissues expressed COX-1, COX-2, and 5-LO, the IAS expressed significantly higher levels of COX-1 (n = 4; Fig. 6). There were no significant differences in the levels of COX-2 and 5-LO in the IAS compared with the RSM (P > 0.05; n = 4; Fig. 6).

mRNA expression of COX-1, COX-2, and 5-LO using RT-PCR analyses. RT-PCR studies were performed to determine the relative levels of COX-1, COX-2, and 5-LO using RNA extracts from IAS and RSM. The IAS expressed significantly higher levels of COX-1 and COX-2 mRNAs (n = 4; Fig. 7). The levels of 5-LO, however, were not significantly different between the two tissues (P > 0.05; n = 4; Fig. 7).

These results suggest significant differences in the translational versus transcriptional levels of these enzymatic systems between the two tissues. The IAS had characteristically higher levels of COX-1 mRNA compared with the RSM (n = 4). On the other hand, COX-2 mRNA levels were only marginally significantly different from the RSM (n = 4). Regarding 5-LO,
there were no significant differences in the levels of 5-LO mRNAs in the IAS versus the RSM (P > 0.05; n = 4). Data with the protein and mRNA expression levels with COX-1 are directly complimentary, showing characteristically higher levels of COX-1 in the IAS. The significance of comparable levels of 5-LO in both tissues remains to be determined.

Effect of COX-1 versus COX-2 selective inhibitors on the effect of AA-induced increase in the IAS tone. To determine the specific nature of COX-1 versus COX-2 in the rapidly induced increase in the IAS tone by AA, we examined the effect of SC-560 and rofecoxib, COX-1 and COX-2 inhibitors (7, 23), respectively. Data show that SC-560 caused concentration-dependent decrease in the AA-induced increase in the IAS tone (n = 4; Fig. 8A). Rofecoxib, on the other hand, had no significant effect on AA-induced increase in the IAS tone (P > 0.05; n = 4; Fig. 8B). These data combined with the higher expression of COX-1 in the IAS suggest that COX-1 is primarily responsible for the AA-induced increase in the IAS tone. However, exact significance of the higher levels of COX-2 mRNA in the IAS remains to be determined.

DISCUSSION

Present studies suggest COX and 5-LO pathways play counter-regulatory roles in the IAS tone. In this regard, although the COX pathway is primarily excitatory, 5-LO may be inhibitory. The data further suggest that the COX pathway is in part responsible for the basal tone in the IAS. The role of COXs and 5-LO in the present studies was determined by the effect of AA and its metabolites and of the selective inhibitors of COXs and 5-LO. In addition, we examined the translational and transcriptional levels of these two distinct enzymatic pathways in the IAS.

Earlier studies from our laboratory suggest the involvement of a local RAS in part for the maintenance of the IAS basal tone via both in vivo (14) and in vitro (11, 13) experiments. Such findings clearly suggest the involvement of additional unknown mechanisms that provide external trigger for the genesis of basal tone. Previous studies in the human and cat LES (5, 6) suggest that AA metabolites produced via COX activity sustain basal tone in the LES. In the present study we specifically examined the role of AA metabolites via COX and 5-LO in the IAS tone. For the direct comparison, we also examined their role in the RSM, which is primarily phasic in nature.

The studies show COX inhibition by indomethacin causes significant and concentration-dependent decrease in the basal tone of the IAS. Second, the overall effect of AA is to increase the IAS tone. AA increased the IAS basal tone in a concentration-dependent manner. AA was significantly more efficacious and potent in the IAS than in the RSM. These effects of AA in the anorectal smooth muscle were selectively mediated via COX as they were virtually abolished by the lower concentrations of the dual COX inhibitor. These data suggest that AA metabolism via COXs contributes significantly toward the genesis of basal tone. Previous studies in the human and cat LES (5, 6) suggest that AA metabolites produced via COX contribute significantly toward the IAS basal tone. To provide further credence to the hypothesis, Western blot and RT-PCR data show that the COX isoform COX-1 is responsible for the production of prostaglandins and thromboxanes. COX-2, on the other hand, has been demonstrated to be involved in the inflammatory or mitogenic processes (26). Western blot and RT-PCR data show that the overall levels of COXs, especially COX-1, in the IAS is significantly higher that in the RSM. These results may provide an explanation for the more efficacious and more potent effects of AA in the IAS versus the RSM.

COX-mediated AA metabolism occurs primarily by two enzyme isoforms: COX-1 and COX-2. The constitutive isoform COX-1 is responsible for the production of prostaglandins and thromboxanes. COX-2, on the other hand, has been demonstrated to be involved in the inflammatory or mitogenic processes (26). Western blot and RT-PCR data show that the overall levels of COXs, especially COX-1, in the IAS is significantly higher that in the RSM. These results may provide an explanation for the more efficacious and more potent effects of AA in the IAS versus the RSM.

AA may also be converted by 5-LO into other physiologically relevant compounds such as LTB4, LTC4, LTD4, LXA4, and 5-HETE (4, 16, 17, 22). Detailed examination reveals that 5-LO inhibitor NDGA causes no significant effect on the IAS tone. Interestingly, however, NDGA partly reverses the indomethacin-decreased IAS tone. Additionally, the predominant effect of 5-LO metabolites (LTC4, LXA4, and 5-HETE) (16) is...
to decrease the IAS smooth muscle tone rather than increase
caused by the COX products. The other metabolites LTB₄ and
LTD₄ do, however, produce marginal increases in the IAS tone
without any significant effect on the RSM force. These data
provide evidence for counter-regulation between the COX and
the 5-LO pathways in the basal tone IAS tone. Additionally,
current data suggest that in contrast with the prominent role of
COX, the role of 5-LO in the IAS tone regulation may be
limited.

Above data suggest that AA may potentially acquire the
course of the excitatory pathways of COX or inhibitory via
5-LO in the IAS. This dual (excitatory and inhibitory) course
that AA may follow versus the unidirectional course of throm-
boxane analog U-46619 may explain relatively lower efficacy
and potency of AA in causing an increase in the IAS tone.

Superficially, similar trends of the effects of AA and metab-
olites may give an impression that these substances possess
similar roles in the IAS and RSM. However, in-depth exami-
nation reveals important differences. The IAS compared with
the RSM has higher levels of COX-1 and COX-2 at both the
transcriptional and translational levels. Furthermore, functional
studies reveal no significant effect of COX inhibition on the
ongoing activity of RSM in contrast with its effect on the basal
tone in the IAS. In addition, preliminary findings show that in
the basal state IAS has characteristically higher levels of AA
and metabolites (especially PGF₂α and TxA₂; unpublished
observations).

Western blots and RT-PCR analyses reveal the presence of
not only COX but also 5-LO in the anorectal tissues. To
determine the functional significance of 5-LO, we examined
the effects of 5-LO inhibition by NDGA. The data show that
although NDGA by itself produces no significant effect, it
partly reverses indomethacin-attenuated IAS tone. We specu-
late that this reversal is because of the underlying portion
of the counter-regulatory inhibitory pathway that is mediated
via 5-LO.

Diverse studies show that COX and 5-LO have different
affinities for AA. Km values for AA in the case of COX and
5-LO range from 1 to 3 μM and from 10 to 20 μM, respec-
tively (24, 25). This difference may partly explain the diver-
gent effects of NDGA, i.e., the lack of a significant effect by
NDGA itself and significant increase in the IAS tone by NDGA
following the COX inhibition. In addition, this difference may
explain the predominantly excitatory role of COX by the
revelation of potent decrease in the IAS tone with COX
inhibition alone. Additionally, it may also explain the lack of
significant effect of NDGA on the overall concentration-re-
sponse curve of AA except at the higher concentrations (300
μM). We speculate lower affinity of 5-LO for AA compared
with COX may primarily direct AA metabolic traffic in the
direction of COX pathway. However, additional experiments
monitoring AA metabolites in the presence of indomethacin
and NDGA may further unravel this issue.

Recent studies from our laboratory have shown that COX-1
is primarily responsible for the basal tone in the IAS (12).
Although this was not the main focus of the studies, following
the lead from those findings, it was considered important to
determine the role of COX-1 versus COX-2 in the AA-induced
increase in the IAS tone. Significant attenuation of the AA
effect by SC-560 and not by rofecoxib suggests that rapid
conversion of AA into excitatory metabolites is primarily
dependent upon the constitutive COX-1 pathway. The data,
however, do not rule out the role of the inducible COX-2
pathway following long-term exposure of AA in the IAS.

In summary, these studies suggest that the biosynthesis of
AA and its conversion into prostanooids by COXs, especially
COX-1, within the smooth muscle cells may be important for
the basal IAS tone. Conversely, 5-LO metabolites may provide
inhibitory modulation of the IAS tone. These data provide
evidence for the counter-regulation between the COX and
the 5-LO pathways in the IAS. The studies have pathophysiological
relevance in the anorectal motility disorders that affect the IAS.

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