COX-1 vs. COX-2 as a determinant of basal tone in the internal anal sphincter

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Prostanoids, produced endogenously via cyclooxygenases (COXs), have been implicated in the sustained contraction of different smooth muscles. The two major types of COXs are COX-1 and COX-2. The COX subtype involved in the basal state of the internal anal sphincter (IAS) smooth muscle tone is not known. To identify the COX subtype, we examined the effect of COX-1- and COX-2-selective inhibitors, SC-560 and rofecoxib, respectively, on basal tone in the rat IAS. We also determined the effect of selective deletion of COX-1 and COX-2 genes (COX-1−/− and COX-2−/− mice) on basal tone in murine IAS. Our data show that SC-560 causes significantly more efficacious and potent concentration-dependent decreases in IAS tone than rofecoxib. In support of these data, significantly higher levels of COX-1 than COX-2 mRNA were found in the IAS. In addition, higher levels of COX-1 mRNA and protein were expressed in rat IAS than rectal smooth muscle. In wild-type mice, IAS tone was decreased 41.4 ± 2.2% by SC-560 (1 μM) and 5.4 ± 2.2% by rofecoxib (P < 0.05, n = 5). Basal tone was 0.172 ± 0.021 mN/mg in the IAS from wild-type mice and significantly less (0.080 ± 0.015 mN/mg) in the IAS from COX-1−/− mice (P < 0.05, n = 5). However, basal tone in COX-2−/− mice was not significantly different from that in wild-type mice. We conclude that COX-1-related products contribute significantly to IAS tone.

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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 smooth muscle strips was anchored at the bottom of the muscle bath, and the other end was connected to a force transducer (model FT03, Grass Instruments, Quincy, MA). Isometric tension was measured by the PowerLab/SP data acquisition system using Chart 4.1.2 (ADInstruments). Each smooth muscle strip was initially stretched to a tension of 1.0 g and then allowed to equilibrate for 60 min. During this equilibration period, the muscle bath was replenished with fresh KPS at 20-min intervals. Only the strips that developed spontaneous tone and relaxed to electrical field stimulation (10 Hz, 20 V, 0.5-ms pulse duration, and 4-s train duration) delivered from a stimulator (model S88, Grass Instruments) were used.

**Drug responses.** First, we determined the effects of cumulative concentrations of indomethacin (0.1–300 μM) on basal tone of the IAS. Next, we examined the effects of selective inhibitors of COX-1 and COX-2, SC-560 and rofecoxib, respectively (both at 0.01–10 μM). The experiments were repeated in the presence of PGF2α and TxA2 receptor antagonists, AL-8810 and SQ-29598 (both 0.1–10 μM), respectively. The decreases in IAS tone induced by the inhibitors were measured and normalized to the maximal decrease in tone induced by 50 mM EDTA, as previously described (14).

**Western blot analysis.** Western blot studies were performed to determine the relative distribution of COX-1 and COX-2 following the previously described method from our laboratory (14). Briefly, after their isolation, IAS and rectum smooth muscle (RSM), which was used as an internal control, were subjected to homogenization and protein extraction. Proteins were determined by the method of Lowry et al. (24) and then separated by gel electrophoresis and transferred onto a nitrocellulose membrane (NCM) at 4°C. Nonspecific binding on the NCM was blocked with nonfat milk (5%) in Tris-buffered saline-Tween 20 [20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20] overnight at 4°C. Then the NCM was incubated with horseradish peroxidase-labeled secondary antibody (1:10,000 dilution) for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substrate using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and Hyperfilm MP (Amersham Biosciences).

The NCM was stripped of antibodies by exposure to Restore Western blot stripping buffer (Pierce) for 10 min at room temperature and then reprobed for α-actin using the specific primary antibody (rabbit anti-COX-1 and goat anti-COX-2 at 1:1,000 dilution) for 2 h at room temperature. After it was washed with Tris-buffered saline-Tween 20, the NCM was incubated with horseradish peroxidase-labeled secondary antibody (1:10,000 dilution) for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substrate using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and Hyperfilm MP (Amersham Biosciences).

Table 1. Primers used in RT-PCRs for amplification of mRNA encoding COX-1, COX-2, and β-actin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1 Forward</td>
<td>S67721</td>
<td>CTCAACAGCTGAATTCAC</td>
</tr>
<tr>
<td>COX-1 Reverse</td>
<td>S67721</td>
<td>CTGACAGTCTAGTTTAT</td>
</tr>
<tr>
<td>COX-2 Forward</td>
<td>S67722</td>
<td>AGCTGTGACTCATGACATAGGA</td>
</tr>
<tr>
<td>COX-2 Reverse</td>
<td>S67722</td>
<td>TGGTCGGTGATGTTCGAGAT</td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>NM_007393</td>
<td>GTGTTGAGACCCCTCCACCC</td>
</tr>
<tr>
<td>β-Actin Reverse</td>
<td>NM_007393</td>
<td>AGCTCACACTGCTGATGAA</td>
</tr>
</tbody>
</table>

COX, cyclooxygenase.
GCC TCT GTT CCA CAT ACA C of the COX-1 wild-type forward primer 5'-AGG AGA TGG CTG CTG AGT TGG-3'
and the reverse primer 5'-ATC CCT TCA CTA AAT GCC CTC-3'. The thermal cycler was programmed for 1 cycle at 94°C for 1 min and 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and was then held at 4°C. The COX-1 wild-type band was 601 bp. The COX-1 wild-type band was 646 bp. The COX-2 wild-type band was 760 bp. The COX-2 band was 905 bp.

Comparison of basal tone in IAS from knockout mice. To further evaluate the role of COX isoforms in IAS tone, isometric force measurements were obtained from IAS strips isolated from the COX-1−/− and COX-2−/− mice and their wild-type counterparts. Data were collected as described above before and after cumulative concentrations of SC-560 or rofecoxib (both at 0.01–10 μM).

Drugs and antibodies. Rofecoxib was obtained from Fisher Scientific (Pittsburg, PA), indomethacin and SC-560 from Sigma-Aldrich (St. Louis, MO), and AL-8810 and SQ-29598 from Cayman Chemical (Ann Arbor, MI). The antibody for α-actin was obtained from Sigma (St. Louis, MO), and all other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Data analysis. Values are means ± SE. Concentration-response curves were analyzed using a nonlinear interactive fitting program (Prism 3.0, Graph Pad Software). Inhibitor potencies and maximum inhibition are expressed as pIC50 (the negative logarithm of the molar concentration of inhibitor producing 50% of the maximum inhibition) and Imax (maximum inhibition elicited by the inhibitor), respectively. Statistical significance was tested by one-way ANOVA followed by Dunnett’s post hoc test when three or more different groups were compared. Student’s t-test was used to compare only two different groups. P < 0.05 was considered statistically significant.

Fig. 2. Effects of COX-1 and COX-2 inhibitors (SC-560 and rofecoxib, respectively) on basal tone in rat IAS. Both inhibitors significantly decrease IAS tone (*P < 0.05). However, SC-560 is more potent than rofecoxib (#P < 0.05). Values are means ± SE (n = 5).

Fig. 3. A and B: RT-PCR shows significantly higher levels of COX-1 and COX-2 in IAS than in rectal smooth muscle (RSM) from rat. Data were normalized to β-actin levels. C and D: Western blot analyses show higher expression of COX-1 in IAS than RSM. Data were normalized to α-actin levels. Values are means ± SE. *P < 0.05.
RESULTS

Effects of indomethacin on basal tone in the IAS. The nonselective COX inhibitor indomethacin produced a concentration-dependent decrease in basal tone in the IAS, with $I_{\text{max}}$ of 71.5 ± 5.2% and pIC$_{50}$ of 5.2 ± 0.1 ($n = 9$). The vehicle (Na$_2$CO$_3$) solution did not produce a significant ($P > 0.05$) effect (Fig. 1 A). An actual trace of the effect of indomethacin on the basal tone in the IAS is shown in Fig. 1 B. Data suggest a significant contribution by COX to IAS tone.

To examine the specific nature of the COX involved in IAS tone, experiments with selective inhibitors were designed to evaluate the relative contribution of COX-1 and COX-2. COX-1 and COX-2 inhibitors, SC-560 and rofecoxib, respectively, produced significant decreases in basal tone in rat IAS ($P < 0.05$, $n = 5$; Fig. 2). SC-560 was significantly ($P < 0.05$, $n = 5$; Fig. 2) more efficacious and potent ($I_{\text{max}} = 29.9 ± 5.7\%$ and pIC$_{50} = 6.7 ± 0.1$, $n = 5$) than rofecoxib ($I_{\text{max}} = 13.5 ± 5.7\%$ and pIC$_{50} = 5.0 ± 0.1$, $n = 4$). These data suggest that COX-1 is the main isoform responsible for maintenance of basal tone in the IAS.

RT-PCR. We compared the relative levels of COX-1 and COX-2 in RNA extracts from rat IAS and RSM. The IAS expressed higher levels of COX-1 and COX-2 than the RSM ($P < 0.05$, $n = 5$; Fig. 3, A and B). Western blots. We also evaluated the presence of COX-1 and COX-2 in the protein extracts obtained from IAS and RSM samples. On the basis of calculations normalized to $\beta$-actin expression, significantly higher levels of COX-1 were expressed in the IAS than in the RSM ($P < 0.05$, $n = 5$; Fig. 3 C). However, differences in COX-2 levels between the IAS and RSM, normalized to $\beta$-actin expression, were not statistically significant ($P > 0.05$, $n = 5$; Fig. 3 D).

Comparison of basal tone in the IAS from knockout mice. COX-1$^{-/-}$ and COX-2$^{-/-}$ mice were identified by genotyping (Fig. 4 A), and differences in tone development by IAS smooth muscle strips isolated from COX-1$^{-/-}$ and COX-2$^{-/-}$ mice would be shown.

Fig. 4. A: RT-PCR products of genomic DNA from COX-1- and COX-2-knockout (COX-1$^{-/-}$ and COX-2$^{-/-}$) and wild-type [WT (COX-1$^{+/+}$ and COX-2$^{+/+}$)] mice. B: IAS from COX-1$^{-/-}$ mice developed significantly lower basal tone than IAS from WT mice. C: there were no significant differences in IAS tone between WT and COX-2$^{-/-}$ mice ($P > 0.05$). Values are means ± SE ($n = 5$). *$P < 0.05$.

Fig. 5. A: SC-560 causes significantly more efficacious, potent, and concentration-dependent decrease in IAS tone in WT than COX-1$^{-/-}$ mice. B: SC-560 causes significant and concentration-dependent decreases in IAS tone not only in the WT, but also in COX-2$^{-/-}$ mice. Values are means ± SE ($n = 4$). *$P < 0.05$. 

Effects of selective inhibitors of COX-1 (SC-560) and COX-2 (rofecoxib) on basal tone in the IAS of wild-type vs. COX-1 and COX-2 mice. The purpose of these experiments was to compare the effects of COX-1- and COX-2-selective inhibitors and to cross-examine the effect of selective deletions of COX-1 and COX-2 genes in the mice on basal tone in the IAS. SC-560 and rofecoxib data from the wild-type mice confirm the significantly less spontaneous tone developed by COX-1 knockout IAS smooth muscle from COX-1-/- mice than by the control group (0.08 ± 0.02 vs. 0.17 ± 0.02 mN/mg, P < 0.05, n = 5; Fig. 4B). Conversely, COX-2 gene deletion did not significantly affect basal tone (P > 0.05, n = 5; Fig. 4C).

In the wild-type mice for COX-1, the COX-1 inhibitor SC-560 (1 × 10⁻⁵ M) produced a significant decrease in IAS tone (41.4 ± 3.4%, P < 0.05, n = 4; Fig. 5A). In COX-1-/- mice, the same concentration of SC-560 produced no significant decrease (5.5 ± 3.9%, P > 0.05; Fig. 5A). These data show that SC-560 was significantly less efficacious and potent (P < 0.05) in the COX-1-/- than in the wild-type mice. These findings further authenticate the selective deletion of the COX-1 gene in these mice. Interestingly, the SC-560-mediated decrease in IAS tone was similar and significant in the COX-2-/- mice, as well as in their wild-type counterparts (P < 0.05, n = 4; Figs. 5B).

Potency comparisons reveal that the COX-2 inhibitor rofecoxib was significantly more potent than rofecoxib in basal tone in WT mice. Higher concentrations of rofecoxib (not shown) may, however, cause a modest decrease in IAS tone.

Effects of selective inhibitors of PGF₂α (AL-8810) and TxA₂ (SQ-29598) receptors on IAS tone. The PGF₂α and TxA₂ receptor-selective inhibitors AL-8810 and SQ-29598, respectively, produced a significant concentration-dependent decrease in IAS tone (P < 0.05, n = 4). SQ-29598 (71.6 ± 9.8%, n = 4) was significantly more efficacious (P < 0.05) than AL-8810 (39.2 ± 2.3%, n = 4; Fig. 7). These data suggest a more important role for TxA₂ than PGF₂α in the IAS.

DISCUSSION

Previous studies from our laboratory showed that sPLA₂, which converts membrane phospholipids to AA within the IAS,
contracts the smooth muscle cells of the IAS, resulting in tone development (16). In the present studies, we show that COX inhibition significantly decreases IAS tone. Using COX-1<sup>−/−</sup> and COX-2<sup>−/−</sup> mice combined with the application of selective inhibitors, we further demonstrate, for the first time, that COX-1 contributes significantly to basal tone in the IAS.

The present studies were carried out in rats and mice. The IAS of rats and mice has characteristics similar to the IAS of humans: it develops spontaneous tone and relaxes in response to nonadrenergic noncholinergic nerve stimulation (7, 29, 37, 38). Our earlier studies suggested that RhoA/ROCK provides the molecular basis for the bulk of the tone in the IAS (28, 30). However, the external trigger(s) that excites the molecular machinery is not exactly known. In earlier studies, we provided evidence for the involvement of a local RAS in the partial (~25%) maintenance of basal tone in rat IAS in vitro (13, 14) and in vivo (15). These data suggest the involvement of other mechanisms.

Using multipronged approaches of functional, molecular biology and genetically modified animals, we evaluated the relative contribution of COXs to basal tone in the IAS. We have shown that nonselective inhibition of COXs by indomethacin and selective inhibition by the COX-1 inhibitor SC-560 cause a significant decrease in basal tone in rat IAS. On the other hand, the selective inhibitor of COX-2 does not significantly decrease basal tone, except at the higher concentrations. Similar data obtained from study of wild-type mice show that SC-560 significantly decreases IAS tone.

Data obtained from COX-1<sup>−/−</sup> mice support the above-stated hypothesis that COX-1, rather than COX-2, plays a major role in regulation of basal tone in the IAS. IAS smooth muscle from COX-1<sup>−/−</sup> mice developed significantly less basal tone than IAS smooth muscle from wild-type mice: 0.17 ± 0.02 vs. 0.08 ± 0.01 mN/mg, which represents a 53% decrease in basal tone in the COX-1<sup>−/−</sup> mice. This decrease is similar to that produced by the COX-1-selective inhibitor in the wild-type mice. On the contrary, the changes in the basal tone of the mice were not significantly different from controls. In addition, rofecoxib has no significant effect on IAS tone in the COX-2<sup>−/−</sup> or wild-type mice.

In rat IAS, the maximal inhibitory effects of the dual COX inhibitor indomethacin (71.4 ± 5.1%) were significantly greater than those of the COX-1-selective inhibitor alone (30 ± 5.6%). One explanation for this finding might be a shared, nonselective inhibitory effect of indomethacin and rofecoxib on basal tone (1); another might be lower efficacy and potency of the COX-1-selective inhibitor SC-560 in rat IAS.

The maximal inhibitory effect of indomethacin observed in the present studies in rat IAS is comparable to that induced by the sPLA<sub>2</sub>-selective inhibitor MJ-33 (66.7%) (16). These data support the notion that basal IAS tone is primarily controlled by a cascade of reactions that modulate the conversion of membrane phospholipids to contractile molecules via sPLA<sub>2</sub> and COX-1 activities, respectively.

RT-PCR and Western blot data confirmed the presence of COX-1, as well as COX-2, in the anorectal region. Translation and transcriptional studies reveal the presence of higher levels of COX-1 in the IAS than RSM. On the basis of functional data, the significance of higher levels of COX-1 in the IAS is self-explanatory.

Collectively, these data suggest that the COX-1 pathway (by the production of smooth muscle contractile prostanoids, such as PGF<sub>2α</sub> and thromboxanes) contributes significantly to basal tone in the IAS. This hypothesis was further validated by the effects of the PGF<sub>2α</sub> and TXA<sub>2</sub> receptor-selective antagonists AL-8810 and SQ-29598, respectively, on IAS tone. These antagonists caused a significant concentration-dependent decrease in IAS tone. SQ-29598 is more potent than AL-8810 (71.6 ± 9.8% vs. 39.2 ± 2.3%, n = 4, P < 0.05), suggesting a more important role of TXA<sub>2</sub> than PGF<sub>2α</sub> in the IAS. Earlier studies showed that PGF<sub>2α</sub> and thromboxanes significantly increase basal tone in LES smooth muscle (5, 9, 31). Consistent with these findings, the COX-1-selective inhibitors and COX-1 small interfering RNA significantly decrease the tone of the LES and gallbladder (4, 5, 9). In the present studies, we did not measure the local levels of PGF<sub>2α</sub>, TXA<sub>2</sub>, or other prostanoids in the IAS. The existing evidence, however, supports our hypothesis that these prostanoids, produced via COX-1, provide an important external trigger for basal tone in the IAS.

Furthermore, it is possible that COX-1 products, along with ANG II, provide a bulk of the signal for IAS tone. We speculate that COX-1 and RAS products may work in concert by activation of RhoA/ROCK and PKC, in definite proportions, to account for the molecular mechanisms for the spontaneous basal tone. RhoA/ROCK and PKC have been suggested to play a major role in the sustained contraction and basal tone of different smooth muscle tissues (2, 19, 27, 30). However, the specific role of RhoA/ROCK and PKC in the COX-1-sustained tone in the IAS remains to be determined.

In summary, the present data suggest that the COX-1 pathway plays a significant role in the maintenance of basal tone in IAS smooth muscle. However, studies of the specific effects of COX-1 products and their specific inhibitors are needed. The present study has important implications for the therapeutic rationale for COX-1-selective inhibitors in the hypertensive IAS associated with anorectal motility disorders.

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**REFERENCES**


