Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans

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Rattan S, Singh J, Kumar S, Phillips B. Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans. Am J Physiol Gastrointest Liver Physiol 308: G924–G933, 2015. First published April 16, 2015; doi:10.1152/ajpgi.00017.2015.—The extracellular signal that triggers activation of rho-associated kinase (RhoA/ROCK), the major molecular determinant of basal internal anal sphincter (IAS) smooth muscle tone, is not known. Using human IAS tissues, we identified the presence of the biosynthetic machineries for angiotensin II (ANG II), thromboxane A2 (TXA2), and prostaglandin F2α (PGF2α). These end products of the renin-angiotensin system (RAS) (ANG II) and arachidonic acid (AA) pathways cause increase in the IAS tone via activation of RhoA/ROCK. Both AA and RAS pathways are known to activate RhoA/ROCK via the G protein-coupled receptors (GPCRs) AT1-R, TXA2 receptor (TPR), and PGF2α receptor (FPR), respectively (1, 50). Recent studies from different laboratories have demonstrated the role of RAS (8, 21, 33, 48) and AA pathways in the contractility of the human gastrointestinal smooth muscle (5, 11, 13, 24). However, to our knowledge, there have been no published data describing the role of RAS and AA pathway on basal muscular tone in human IAS.

In consideration of study of the RAS and AA pathway, a number of inhibitors have been identified that specifically interfere with various pathway steps. In the RAS, renin converts angiotensinogen into ANG I, which is then converted to ANG II via angiotensin-converting enzyme (ACE) (20, 39). H-77 and captopril are potent inhibitors of renin and ACE, respectively (9, 17, 18). SQ29548 and AL-8810 are competitive TPR and FPR antagonists, respectively (17). Because rofecoxib (9) is a selective inhibitor of COX-1 and COX-2, domethacin (26) is a dual COX inhibitor, and SC-560 and mophenacylbromide (47). Cyclooxygenases (28) (COX-1 and COX-2) in turn catalyze the conversion of AA to prostanooids such as PGF2α and TXA2 (34). BPB (4-bromophenacylbromide) is a nonselective inhibitor of PLA2. Indomethacin (26) is a dual COX inhibitor, and SC-560 and rofecoxib (9) are selective inhibitors of COX-1 and COX-2, respectively (9, 17, 18). SQ29548 and AL-8810 are competitive TPR and FPR antagonists, respectively (17). Because TXA2 is an unstable compound, U46619 serves as the stable analog of TXA2 (10).

The purpose of the present investigation is to systematically determine the presence of biosynthetic machinery, the effects of the end products, and of counterpart inhibitors of the RAS and AA pathways in the basal IAS tone. In addition, our studies sought a link between the extracellular signal related to these pathways and RhoA/ROCK II signal transduction cascade. Because of the important role of IAS in multiple rectoanai disorders, such information will have significant pathophysiological and therapeutic implications.
**MATERIALS AND METHODS**

**Tissue preparation.** Tissue samples of internal anal sphincter (IAS) and rectal (RSM) smooth muscles from ten human subjects (from adult men as well as women) who underwent surgical resection of anorectum, were provided by the departments of surgery and pathology. None the subjects were on any of the medications that could directly or indirectly affect AA or RAS pathway. All tissue specimens were assessed to be normal and free of any inflammatory conditions. The studies were approved by the Institutional Review Board of Thomas Jefferson University. Tissue samples were obtained in the operating room and immediately placed in oxygenated, 0.22 μM filtered Krebs physiological solution (KPS) at 4°C. The composition of KPS was (in mM) 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃ and 11.10 glucose. The IAS and rectal tissues were identified and cleaned of the extraneous serosal and mucosal tissues by an experienced colorectal surgeon using sharp dissection, and tissue identification was confirmed by functional studies.

**Measurement of isometric tension.** The smooth muscle strips were transferred to 2-ml muscle baths containing oxygenated KPS at 37°C. Isometric tension was measured with force transducers (FORT10, WPI, Sarasota, FL), and a PowerLab/SPS data-acquisition system (AD Instruments, Colorado Springs, CO) was employed, as described before (43).

**Effects of different agents were examined as cumulative concentration (1 nM to 10 μM)-response curves (CRC) as previously explained (15, 46), before and after different antagonists. In certain experiments, we also examined the effects of the inhibitors given in different combinations as outlined in RESULTS.**

**Isolation of SMCs and cell culture.** Smooth muscle cells (SMCs) from the IAS and RSM were isolated as described previously (38, 43, 45). Briefly, the circular smooth muscle tissues were minced into ~1-mm cubes with sharp scissors and kept for incubation in oxygenated KPS (containing 0.1% collagenase type I and 0.01% soybean trypsin inhibitor) at 37°C. The cell suspension was filtered through a 500-μM Nitex mesh. The filtrate (in collagenase-free KPS) containing 500–H9262/11011 (45). Briefly, the circular smooth muscle tissues were minced into experiments, we also examined the effects of the inhibitors given in 4°C for appropriate polymerization of the mounting medium and then sealed with clear nail polish. Microscopic images were taken on a Carl Zeiss LSM 510 UV META inverted confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) using a Plan-Apo 40 oil immersion lens (at RT) and Zeiss AIM 4.2 SP1 software (Bioimaging Facility of the Kimmel Cancer Center, Thomas Jefferson University). Images were analyzed for immunofluorescence intensity (IFI) by use of Nikon imaging software (NIS elements 3.1) (Melville, NY).

**Particulate and cytosolic fractions isolation.** The IAS and RSM smooth muscle strips were flash frozen by using a Wollenberger clamp (immersed in liquid N₂), before and after maximally effective concentrations of different agents. The frozen tissues were homogenized in ice-cold homogenization buffer (10 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM EDTA, 250 mM sucrose, and 1 mM dithiothreitol). The homogenates were centrifuged at 100,000 g for 30 min at 4°C (Beckman L8-70M Ultracentrifuge: Beckman Coulter, Fullerton, CA). The supernatants were then transferred to a fresh tube and used as the cytosolic fractions. The pellets were resuspended and homogenized in buffer containing 1% Triton X-100. The pellet extract was centrifuged at 800 g for 10 min, and protein concentrations in the resultant supernatants were determined by use of a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) (45).

**Western blot studies.** Protein (30 μg) was mixed in 30 μl of lysates with Labsolv sample buffer (with final concentrations of 62.5 mM Tris, 1% SDS, 15% glycerol, 0.005% bromophenol blue, and 2% mercaptoethanol) and placed in boiling water bath for 5 min. Proteins in the samples were separated by SDS-PAGE gel [7.5% gel for ACE, COX-1, COX-2, ROCK II, and phosphorylated form of MYPT1; 10% gel for renin, AT₁-R, TPR, FPR, and RhoA; 15% gel for myosin light chain (MLC₂₀) and phosphorylated form of MLC₂₀ (pThr18/Ser19-MLC₂₀) and electrophoretically transferred on to a polyvinylidene difluoride membrane by use of the IBlot Dry Blotting System (Invitrogen, Carlsbad, CA) at RT. The membrane was soaked for 1 h at RT in LI-COR buffer, following which it was incubated overnight with primary antibodies (1:1,000 for ACE, COX-1, COX-2, renin, AT₁-R, TPR, FPR, RhoA, ROCK II, p-MYPT1, MYPT1, MLC₂₀, and p-MLC₂₀, and 1:20,000 for α-actin) diluted in LI-COR buffer containing 0.1% Tween 20. After washing three times for 10 min each with Tris-buffered saline with Tween 20 (TBS-T) the membranes were incubated in dark for 1 h, respectively with IRdye680 and IRdye800 conjugated secondary antibody from LI-COR Biosciences (Lincoln, NE). The membranes were washed three times for 10 min each with TBS-T and finally kept in PBS buffer on shaker for 10 min at RT in the dark and scanned by LI-COR Infrared scanner (LI-COR Biosciences).

**Chemicals and reagents.** ANG II, captopril, indomethacin, Sc-560, BBP, PGE₂, α-actin, and MLC₂₀ primary antibody were obtained from Sigma (St. Louis, MO). Rofecoxib, AL8180, U46619, and SQ29548 were from Cayman Chemical (Ann Arbor, MI). Y27632 was purchased from Biomol (Plymouth Meeting, PA). H77 was from Bachem (Bubendorf, Switzerland). Losartan was a gift from Merck (Rahway, NJ). Primary antibodies of renin, ACE, COX-1, COX-2, ROCK II, p-Thr680-MYPT1, MYPT1, MLC₂₀, and p-MLC₂₀, and 1:20,000 for α-actin) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IRdye680- and IRdye800-conjugated secondary antibodies against mouse, goat, and rabbit were from Santa Cruz Biotechnology (Santa Cruz, CA). IRdye680- and IRdye800-conjugated secondary antibodies against mouse, goat, and rabbit were from LI-COR Biosciences.

**Data analyses.** Results are expressed as means ± SE of four to eight independent determinants. Analysist and antagonist CRCₘₜₜ in % maximal responses and calculations of potencies were made by using Prism 5.1 (GraphPad Software). All relaxation and contractile responses were calculated on the bases of % maximal responses with 800 μM ANG II, U46619 (10 μM), respectively.

IFIs were calculated by use of NIS Elements 3.1 software (Melville, NY) and plotted as bar graph analyses. For this, total intensity (pixels/area) across the cells was calculated by drawing a line across the cell avoiding the nucleus, at four different sites, as described before (44). Statistical significance was determine by one-way ANOVA followed by Dennett’s comparison test. Unpaired Student’s
t-test was used to compare two different groups. A P value < 0.05 was considered statistically significant.

RESULTS

Effects of ANG II on basal tone in human IAS. ANG II (1 nM to 1 μM) produced concentration-dependent increase in the IAS tone, with maximal increase of 19.1 ± 1.6% (Fig. 1A). Cross-reactivity protocols revealed a selective and significant antagonism (*P < 0.05; n = 8) of ANG II CRC by the AT1-R antagonist losartan and not by the TPR antagonist SQ29548.

Effects of U46619 and PGF2α on basal tone in human IAS. Stable analog of TXA2 and TPR agonist U46619 also produced...
concentration-dependent increase in the IAS tone, with significantly greater efficacy and potency than ANG II. Normalized at 100% increase in the IAS tone with U46619, maximal effect with ANG II was 20% increase in the IAS tone whereas that with U46619 and with PGF$_{2\alpha}$ was 100 and 65%, respectively (Fig. 1, A–C). Comparison of EC$_{20}$ values (for ANG II $3 \times 10^{-8}$ M, and for U46619 and PGF$_{2\alpha}$ $1 \times 10^{-8}$ M) revealed that U46619 and PGF$_{2\alpha}$ were 30 times more potent than ANG II. Cross-reactivity experiments showed selective and significant rightward shift in the CRC with SQ29548 ($^*P < 0.05; n = 8$; Fig. 1B) and not with losartan ($P > 0.05; n = 8$; Fig. 1B). These data suggest selective activation of TPR by U46619 and U46619 and PGF$_{2\alpha}$.

These inhibitors produced concentration-dependent and significant decrease in the IAS tone. These inhibitors (with the exception of renin inhibitor H-77) produced a limited decrease in the IAS tone: ANG II and U46619 and PGF$_{2\alpha}$ were 30 times more potent than ANG II. These inhibitors produced concentration-dependent decrease in the IAS tone. These inhibitors produced concentration-dependent decrease in the IAS tone. These inhibitors produced concentration-dependent decrease in the IAS tone. These inhibitors produced concentration-dependent decrease in the IAS tone.

Effects of AA pathway inhibitors (nonselective PLA$_2$ inhibitor AL8810, dual COX inhibitor indomethacin, COX-1 inhibitor SC-560, COX-2 inhibitor rofecoxib, TPR antagonist SQ29548, and FPR antagonist AL8810), on human IAS tone. These inhibitors of prostanoid production (Fig. 2) and respective antagonists of TXA$_2$ and PGF$_{2\alpha}$ (Fig. 2C) produced concentration-dependent and significant decrease in the IAS tone ($^*P < 0.05$; $n = 8$).

BPB and indomethacin produced 67.0 ± 4.0 and 83 ± 6.0% decreases in the IAS tone. These values plotted in Fig. 2B were 33.0 ± 4.0 and 17.0 ± 6.0% of basal IAS tone, respectively. Although Sc-560 (COX-1 inhibitor) and rofecoxib (COX-2 inhibitor) individually caused only 18.0 ± 3 and 28.5 ± 5% decrease in IAS tone, respectively, their combination produced ~80% decrease in the IAS tone (data not shown).

SQ29548 and AL8810 in the maximally effective concentrations induced 49.0 ± 3 and 65 ± 8.60% decrease in IAS
Combined effects of AT1-R and TPR antagonists (losartan and SQ29548), and TPR and FPR antagonists (SQ29548 + AL8810), on the basal tone. In the maximally effective concentrations (10 μM), the combined effect of losartan and SQ29548 was 65.0 ± 5% decrease in the IAS tone (or as shown in Fig. 3A, % of basal IAS tone following the combination was 37.0 ± 4.0). Individually, these antagonists produced 24.4 ± 3.5 and 42.0 ± 5% decrease in the IAS tone (or resulted in 75.6 ± 5.0 and 58.9 ± 5.9% basal tone as shown in Fig. 3A), respectively.

Similarly, in fresh series of experiments, SQ29548 and AL8810, when given individually and in combination produced 39.0 ± 3, 57.0 ± 8.6, and 75.0 ± 7.27% decrease in the IAS tone (leading to 60.9 ± 4.3, 42.7 ± 7.6, and 25.2 ± 7.2% basal tone; n = 6 – 8; Fig. 3B), respectively.

Values with the residual basal IAS tone (on percentile basis) following the maximal effect of RAS and AA pathway inhibitors administered either individually or in different combinations are given in Table 1.

On the basis of the above data, the AA pathway appears to provide a more potent extracellular signal for the basal tone in human IAS compared with the RAS pathway (Fig. 3, A and B).

Table 1. Effects of different inhibitors on basal tone in human IAS

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mechanism of Action</th>
<th>% of Basal Tone</th>
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<tr>
<td>H-77</td>
<td>renin inhibitor</td>
<td>52.9 ± 2.0</td>
</tr>
<tr>
<td>Captopril</td>
<td>ACE inhibitor</td>
<td>82.7 ± 2.0</td>
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<tr>
<td>Losartan</td>
<td>AT1-R antagonist</td>
<td>75.6 ± 3.0</td>
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<tr>
<td>BPB</td>
<td>PLA2 inhibitor</td>
<td>33.0 ± 4.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>nonselective COX inhibitor</td>
<td>17.0 ± 6.0</td>
</tr>
<tr>
<td>Sc-560 + Rofecoxib</td>
<td>COX-1 + COX-2 inhibitors</td>
<td>20.0 ± 1.7</td>
</tr>
<tr>
<td>SQ29548</td>
<td>TPR antagonist</td>
<td>50.9 ± 4.0</td>
</tr>
<tr>
<td>AL8810</td>
<td>FPR antagonist</td>
<td>35.2 ± 5.7</td>
</tr>
<tr>
<td>Losartan + SQ29548</td>
<td></td>
<td>37.0 ± 4.0</td>
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<tr>
<td>SQ29548 + AL8810</td>
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<td>25.2 ± 7.2</td>
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Values represent the residual % basal internal anal sphincter (IAS) tone (means ± SE) following the maximal effect of different inhibitors given either individually or in different combinations. Data given in RESULTS represent the absolute decrease in the IAS tone following these treatments. ACE, angiotensin-converting enzyme; BPB, 4-bromophenacylbromide; COX, cyclooxygenase; FPR, prostaglandin F2α receptor; TPR, TXA2 receptor.

Values represent the residual % basal internal anal sphincter (IAS) tone (means ± SE) following the maximal effect of different inhibitors given either individually or in different combinations. Data given in RESULTS represent the absolute decrease in the IAS tone following these treatments.

Effects of ANG II and U46619 in the IAS vs. RSM. To determine whether stimulation of the RAS and AA pathways yields increased tonicity uniquely in the IAS, a comparison was made between the circular muscle layer of the rectum and the circular muscle layer of the IAS when exposed to agonists of these pathways. This comparison of the AT1-R agonist (ANG II) and TPR agonist (U46619) revealed significantly higher expression of RAS pathway components (renin, ACE, and AT1-R) in the IAS vs. RSM. Bottom: ICC data of IFI showing significantly higher expression (*P < 0.05; n = 4) of AA pathway components (COX-1, COX-2, TPR, and FPR) in the IAS vs. RSM.

Fig. 4. Top: immunocytochemistry (ICC) data of relative fluorescence intensities (IFI) showing significantly higher expression (*P < 0.05; n = 4) of RAS pathway components (renin, ACE, and AT1-R) in the IAS vs. RSM. Bottom: ICC data of IFI showing significantly higher expression (*P < 0.05; n = 4) of AA pathway components (COX-1, COX-2, TPR, and FPR) in the IAS vs. RSM.
higher responses in the IAS vs. RSM to either agonist (*P < 0.05; n = 6–8; Fig. 3, C and D). Once again, a more significant increase in IAS tone is seen with stimulation of the AA pathway (U46619).

Relative distribution of renin, ACE, AT1-R, COX-1, COX-2, TPR, and FPR in human IAS vs. RSM SMCs. Immunocytochemistry analysis demonstrated significantly higher IFI/unit area of the IAS vs. RSM SMCs for renin, ACE, and AT1-R (*P < 0.05; n = 4; Fig. 4, top). Similar data were obtained with the IFI for COX-1, COX-2, TPR, and FPR in the IAS vs. RSM SMCs (*P < 0.05; n = 4; Fig. 4, bottom).

Likewise, Western blot data revealed significantly higher expression (*P < 0.05; n = 4) of renin, ACE, and AT1-R (Fig. 5A) and of COX-1, COX-2, TPR, and FPR (Fig. 5B) in the IAS vs. RSM.

These data insinuate a more significant role of the RAS and AA pathways in the mechanics of IAS compared with RSM.

Protein expression of RhoA/ROCK II in response to ANG II, U46619, and PGF2α stimulation in human IAS. Western blot data revealed that ANG II, U46619, and PGF2α significantly increased the expression of both RhoA (Fig. 7A) and ROCK II (Fig. 7B) in the particulate compared with the cytosolic fractions. This increase was attenuated by pretreatment of the cells with Y27632 (*P < 0.05; n = 5).

These data provide further support for the role of RhoA/ROCK in the mediation of basal tone via both the RAS and AA pathways. Translocations of RhoA/ROCK II to the membrane (captured in particulate fractions) are known markers for the activation of RhoA/ROCK pathway (25, 38).

**DISCUSSION**

For the first time (regardless of the species), our study has been able to demonstrate that the end products of AA and RAS pathways, TXA2, PGF2α, and ANG II, provide extracellular signals for the activation RhoA/ROCK for the maintenance of the basal tone in the IAS. The studies have direct relevance in the human pathophysiology of the IAS because they were conducted in human tissues. Data demonstrate that myogenic tone in intact human IAS is regulated via the autocrine control of AA (~80%; via thromboxane A2 and PGF2α) and RAS (~20%; via ANG II) pathways. TXA2, PGF2α, and ANG II are synthesized within the IAS SMCs and subsequently released, resulting in the activation of their respective GPCRs TPR, FPR, and AT1-R. Such activation leads to the stimulation of RhoA/ROCK, causing increase in p-MYPT1 and p-MLC20 that is ultimately responsible for the basal IAS tone.

It is noteworthy that, although the overall control mechanism for the human IAS tone is similar to that in rats (primarily via AA vs. RAS) (15, 17), certain differences exist. According to the previously published studies, rat IAS tone is primarily mediated via TXA2. In those studies, SQ29548 (TPR antagonist) was more efficacious than AL-8810 (FPR antagonist) in reducing the basal IAS tone (15). Conversely, in humans, the
IAS tone is regulated predominantly via PGF\textsubscript{2\alpha} generation and FPR activation. It is evident from the present data that the FPR antagonist AL-8810 is more efficacious than the TPR antagonist SQ29548 in producing decrease in the IAS tone. Additionally, in contrast with rat IAS studies (17, 18), there are significant differences in the nature of PLA\textsubscript{2} and COX in the relative contribution of basal tone in human IAS.

Although the exact nature of PLA\textsubscript{2} and COX in the human IAS tone is not known, it appears that rather than a single isoform [unlike the secreted isoform of PLA\textsubscript{2} (sPLA\textsubscript{2}) and COX-1 in the case of rat], in human IAS multiple subtypes of PLA\textsubscript{2} and COX may be involved. Not shown, in human IAS sPLA\textsubscript{2}-specific inhibitor MJ33 in contrast with the rat studies (18) produces only ~15\% whereas nonselective PLA\textsubscript{2} inhibitor BPB produces 67\% decrease in the tone. Likewise, in contrast with the limited effect of the individual use of COX-1 and COX-2 inhibitors, their combined use and the dual COX inhibitor indomethacin produces a profound decease in IAS tone in humans.

The conclusion for the AA and RAS regulation of the basal tone in human IAS is based on the presence of biosynthetic machinery for these pathways, the effects of their end products (prostanoids TXA\textsubscript{2} and PGF\textsubscript{2\alpha}) and ANG II, respectively, and their mechanistic convergence on the common RhoA/ROCK signal transduction (43). Finally, selective inhibition of the respective pathways causes corresponding decrease in the basal tone in intact human IAS.

Immunocytochemistry and Western blot studies reveal discrete presence of the biosynthetic machinery for both RAS (renin, ACE, and AT\textsubscript{1}-R) and AA (COX-1,2, TPR, and FPR) in the human IAS. Functional data reveal that natural ligands of RAS (ANG II) and AA (TXA\textsubscript{2} and PGF\textsubscript{2\alpha}) produce concentration-dependent increase in the IAS tone, selectively antagonized by their respective antagonists. These data, combined

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**Fig. 6.** Data show that ROCK inhibitor Y27632 causes concentration-dependent and significant inhibition (\(^*P<0.05; n=8\)) of increases in the IAS tone produced by ANG II (A), U46619 (B), and PGF\textsubscript{2\alpha} (C).

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**Fig. 7.** Western blots and corresponding quantitative data show that ROCK inhibitor Y27632 (Y2) significantly inhibits \((\star P<0.05; n=5)\) increases in expressions of RhoA (A) and ROCK II (B), in the particulate fractions caused by ANG II, U46619 (U4), and PGF\textsubscript{2\alpha}. P, particulate; c, cytosolic; Rel., relative.
with the specific decrease in the IAS tone following the selective inhibition and higher expression of AT1-R, TPR, and FPR and severalfold more potent excitatory effects of their agonists in the IAS vs. RSM, suggest the role of RAS and AA pathways in the basal tone in human IAS. The present data show that PGF2\_
\_α and TXA2 contribute to the majority (\~80%) of the IAS tone, whereas the contribution by ANG II may be limited to \~20%. This notion is supported by the limited decrease (\~20%) in the IAS tone by the RAS inhibitors, compared with \~80% decrease in the IAS tone following AA pathways inhibitors.

The unexpected higher efficacy of H-77 (in contrast with the smaller effect by the inhibition of other components of RAS) on the basal IAS tone may be because of nonselective wide spectrum effect with renin blockade (23). Likewise, limited effects of COX-1 and COX-2 inhibitors given alone in the human IAS tone may be explained on the basis of the cross talk between the two, and possibly by the compensatory upregula-

### Nature of Extracellular Signal for the Basal Tone in Human IAS

![Diagram showing the nature of extracellular signal for the basal tone in human IAS](image)

Fig. 9. Model showing that the basal tone in human IAS is mediated via the autocrine control of AA (via thromboxane A2 and PGF2α) and RAS (via ANG II) pathways. ANG II is synthesized from angiotensinogen with the involvement of renin and ACE, respectively, inhibited by H77 and captopril. TXA2 and PGF2α are synthesized via PLA2 and COX-1 and COX-2. As listed, the inhibitors of the AA pathways along different steps are BPB, Sc-560, rofecoxib, indomethacin, SQ2958, and AL1180. Following their release, TXA2 and PGF2α, activate their respective G protein-coupled receptors (GPCR) TPR, FPR, and AT1-R and recruit their specific G proteins (which in the IAS per se are not presently known). This leads to the activation of RhoAROCk, causing increase in the p-MYPT1 and inhibition of myosin light chain phosphatase. These events lead to increase in p-MLC\_20, responsible for the basal tone in the IAS, Y27632 as indicated is a well-known inhibitor of ROCK.
tion of one subtype of COX following inhibition of the other. This notion is supported by the profound decrease in the IAS tone following dual COX inhibitor indomethacin and by the combined use of rofecoxib and Sc-560. In further support of this, as stated above, other AA pathway inhibitors (e.g., non-selective PLA2 inhibition) and combined use of TPR and FPR antagonists also produce ~80% decrease in the IAS tone. Together, these data support the concept of a predominant role of AA pathway in the IAS tone. Further decreases in the IAS tone with the combined use of both RAS and AA pathway inhibition (by losartan and SQ29548) suggest that these pathways may lie in parallel.

Functional and Western blot data show that the ROCK-selective inhibitor Y27632 significantly blocks increase in the IAS tone caused by ANG II, U46619, and PGF2α. Evidence for the predominant role of RhoA/ROCK is also supported by the data wherein ANG II, U46619, and PGF2α produce significant displacement of RhoA/ROCK from the cytosol to the membrane. Such translocations of RhoA/ROCK serve as important markers for RhoA/ROCK activation (37, 38). Activation of RhoA/ROCK increases the levels of p^Thr189-MYPT1 and p^Thr18/Ser19-MLC20. Since basal IAS tone in humans is predominantly mediated via RhoA/ROCK (43), we surmise that these agents utilize RhoA/ROCK for the basal IAS tone.

In support of the present data, recent studies have reported pathophysiological and therapeutic significance of RAS and AA pathways in the esophageal achalasia (7, 8) and in decreased colonic motility in patients with slow-transit constipation on the basis of lower levels of TXA2, PGF2α, and COX-1 in the colon smooth muscle (11). The data that we have presented here in humans have direct implications in the SMC-targeted, novel, and safer therapy of hyper- and hypotonic IAS. Current treatment for hypertonic IAS diseases is limited to available approaches such as Ca2+-channel blockers, NO donors, and phosphodiesterase inhibitors (35, 49). Similarly, in the case of FI associated with the hypotonic anal sphincter, stimulation of AT1-R, TPR, and FPR provides potential novel therapeutic avenues. Moreover, in case of anorectal motility disorders the distinct advantage of topical anal application of 5% captopril (29). It is our expectation that this study’s breakdown and delineation of the pathways leading to basal tone of the internal anal sphincter via the SAS and AA pathways in humans will have real-world implications in the treatment of patients with both hypertonic and hypotonic anorectal diseases.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
All authors contributed equally toward conception and design of research, data collection and interpretation, preparation of figures, drafting, editing, revision, and approval of final version of the manuscript.

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