Bimodal effect of oxidative stress in internal anal sphincter smooth muscle

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Singh J, Kumar S, Rattan S. Bimodal effect of oxidative stress in internal anal sphincter smooth muscle. Am J Physiol Gastrointest Liver Physiol 309: G292–G300, 2015. First published July 2, 2015; doi:10.1152/ajpgi.00125.2015.—Changes in oxidative stress may affect basal tone and relaxation of the internal anal sphincter (IAS) smooth muscle in aging. We examined this issue by investigating the effects of the oxidative stress inducer 6-anilino-5,8-quinolinedione (LY-83583) in basal as well as U-46619-stimulated tone, and nonadrenergic, noncholinergic (NANC) relaxation in rat IAS. LY-83583, which works via generation of reactive oxygen species in living cells, produced a bimodal effect in IAS tone: lower concentrations (0.1 nM to 10 µM) produced a concentration-dependent increase, while higher concentrations (50–100 µM) produced a decrease in IAS tone. An increase in IAS tone by lower concentrations was associated with an increase in RhoA/Rho kinase (ROCK) activity. This was evident by the increase in Rhoa/Rock in the particulate fractions, in ROCK activity, and in the levels of phosphorylated (p) Thr696-myosin phosphatase target subunit 1 and pThr18/20-Ser19-20-kDa myosin light chain. Conversely, higher concentrations of LY-83583 produced inhibitory effects on Rhoa/ROCK. Interestingly, both the excitatory and inhibitory effects of LY-83583 in the IAS were reversed by superoxide dismutase. The excitatory effects of LY-83583 were found to resemble those with neuronal nitric oxide synthase (nNOS) inhibition by l-NNa, since it produced a significant increase in the IAS tone and attenuated NANC relaxation. These effects of LY-83583 and l-NNa were reversible by l-arginine. This suggests the role of nNOS inhibition and Rhoa/ROCK activation in the increase in IAS tone by LY-83583. These data have important implications in the pathophysiology and therapeutic targeting of rectoanal disorders, especially associated with IAS dysfunction.

Increased oxidative stress in aging leads to increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under normal circumstances, ROS and RNS are produced during aerobic cellular metabolism. However, with aging, cumulative effects of excessive ROS and RNS may lead to irreversible changes in cellular function (10), secondary to excessive protein oxidation. This leads to functional decline of the target proteins, which is detrimental to the cells (17, 40).

It has been reported that a moderate level of oxidative stress, generated from protein or lipid oxidation, may be beneficial for cell survival (3, 6, 19). Major ROS/RNS affecting smooth muscle function may be superoxide anion (O2·−) and peroxynitrite (ONOO−). O2·− binds to NO thus producing ONOO−. The latter may affect the cellular functions via protein oxidation or lipid oxidation, leading to permanent cellular damage (27). The body’s antioxidant defense mechanisms, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and heat shock proteins, play a crucial counterregulatory role in ROS and RNS levels. SOD detoxifies O2·− by dismutation into H2O2 while catalase breaks down H2O2 into H2O and O2 (9). Consequently, a decrease in these defense barriers with age may lead to the weakening of the antioxidant defense mechanism, making the cellular compartments vulnerable to free radicals. The role and effects of different levels of oxidative stress in smooth muscle function, specifically in the IAS, have not been examined before.

There is also a gap in the knowledge examining the effects and mechanism of actions of varying grades of oxidative stress on IAS tone. Previously published studies from our laboratory (43) examining the effect of extreme oxidative stress [induced by a high concentration of 6-anilino-5,8-quinolinedione (LY-83583)] revealed a frank decrease in IAS tone, via inactivation of Rhoa/Rho kinase (ROCK) II. LY-83583 is an oxidative stress inducer that works by generating ROS in living cells (11). LY-83583 in lower concentrations (1–10 µM) has been reported to cause tissue-specific contraction (rat pulmonary artery) or relaxation (rat mesenteric artery) in precontracted tissues (46). According to these studies, LY-83583-induced contraction has been shown to be via Rhoa/ROCK, while relaxation may be mediated via voltage-gated K+ channels. Both of these effects were inhibited by SOD and catalase. However, systematic studies examining the effects of different levels of oxidative stress induced via LY-83583 in the spontaneously tonic smooth muscle tissues have not been performed.

The purpose of the present studies is to examine the effects and underlying mechanisms of variable levels of oxidative stress on the IAS tone. We monitored the levels of stress using dihydroethidium (DHE) immunofluorescence intensity (IFI) in relation to the IAS tone, and, for the mechanisms, we monitored changes in Rhoa/ROCK activity and related signal transduction cascade. These studies have important implica-
tions in the pathophysiology and therapeutic targeting in rectoanal motility disorders associated with IAS dysfunction.

MATERIALS AND METHODS

**IAS smooth muscle tissue preparation.** Adult Sprague-Dawley rats were used in the study. Rats were killed by decapitation, and their anal canals were quickly removed and transferred to oxygenated (95% O₂-5% CO₂) Krebs physiological solution (KPS) of the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose (37°C). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

**Force measurement and electric field stimulation.** IAS smooth muscle was carefully identified and separated from the anal canal. The serosa, adventitia with blood vessels, longitudinal smooth muscle layer, and mucosa and submucosa were carefully removed using sharp dissection. Following this, ~1 × 7-mm strips of the circular smooth muscle (CSM) layer were prepared and tied at both ends using thread. These smooth muscle strips were transferred in 2-ml muscle baths where they were anchored to the bottom of the bath at one end and the force transducers (FORT10; WPI, Sarasota, FL) at the other end. These smooth muscle strips were continuously perfused with oxygenated KPS. Initially, 1.0 g of tension was applied, and strips were allowed to equilibrate for 60 min, with repeated washing with fresh KPS. Finally, the cell suspension was filtered through a 500-μm Nitex mesh. The tissue trapped on the mesh was rinsed with 25 ml (5 × 5 ml) of collagenase-free KPS and incubated in collagenase-free KPS at 37°C. The filtrate containing the cells was centrifuged at 350 g for 10 min at room temperature (RT). The cells in the pellet were resuspended on collagen-coated plates in DMEM with 5% fetal bovine serum, 5% penicillin-streptomycin, 50 μg/ml gentamicin, 2 μg/ml amphotericin B, and 50 μg/ml sodium ascorbate (2) in 100-μm tissue culture dishes (Corning) at 37°C and 5% CO₂ in an incubator with regulated humidity.

**Western blot analysis.** IAS smooth muscle strips and SMCs from rats, before and after treatment with LY-83583, were flash-frozen in liquid N₂, suspended in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, and 1% Triton X-100), and homogenized using an IKA Ultra-Turrax T8 tissue homogenizer (Werke, Germany). The extracts were centrifuged as described in Ref. 37, and protein concentration in the resultant supernatant was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Twenty micrograms of protein in 20 μl of lysates were mixed with 2× Laemmli sample buffer (with final concentrations of 62.5 mM Tris, 1% SDS, 15% glycerol, 0.005% bromophenol blue, and 2% mercaptoethanol) and placed in a boiling water bath for 5 min. Proteins in the samples were separated by SDS-polyacrylamide gel electrophoresis (6.5–20% gel with a wide concentration range of LY-83583 on the IAS tone, before and after different activators and inhibitors. We also determined the effect of LY-83583 and L-NNA on different frequencies of electrical field stimulation (EFS, 0.5–20 Hz, 0.5-ms pulse, 12 V, 4-s train duration), before and after L-arginine. IAS smooth muscle strips were identified by development of steady basal tone that responded to immediate relaxation following EFS, and 0Ca²⁺.

The concentration-response curves (CRC) with a wide concentration range of LY-83583 on the IAS tone, before and after different activators and inhibitors. We also determined the effect of LY-83583 and L-NNA on different frequencies of electrical field stimulation (EFS, 0.5–20 Hz, 0.5-ms pulse, 12 V, 4-s train duration), before and after L-arginine. IAS smooth muscle strips were identified by development of steady basal tone that responded to immediate relaxation following EFS, and 0Ca²⁺. All responses showing increase and decrease in the IAS tone were measured and normalized by the maximal contraction induced by 1 μM U-46619, and maximal relaxation with 0Ca²⁺, respectively, in the beginning of the each experiment.

**Isolation of smooth muscle cells and cell culture.** Smooth muscle cells (SMCs) from the IAS were isolated as described previously (37, 42, 44). Briefly, IAS tissues from the CSM layer from adult rats were cut into ~1-mm cubes, and the SMCs were dispersed using repeated short-term incubations with oxygenated KPS containing 0.1% collagenase type I and 0.01% soybean trypsin inhibitor at 37°C for 1 h. Finally, the cell suspension was filtered through a 500-μm Nitex mesh. The tissue trapped on the mesh was rinsed with 25 ml (5 × 5 ml) of collagenase-free KPS and incubated in collagenase-free KPS at 37°C. The filtrate containing the cells was centrifuged at 350 g for 10 min at room temperature (RT). The cells in the pellet were resuspended on collagen-coated plates in DMEM with 5% fetal bovine serum, 5% penicillin-streptomycin, 50 μg/ml gentamicin, 2 μg/ml amphotericin B, and 50 μg/ml sodium ascorbate (2) in 100-μm tissue culture dishes (Corning) at 37°C and 5% CO₂ in an incubator with regulated humidity.

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buffer containing 0.1% Tween 20 for 1 h at RT. After three 10-min wash cycles in Tris-buffered saline-Tween 20, the membranes were incubated with the IRDye680- and IRDye800-conjugated secondary antibody (LI-COR Biosciences) in dark [bovine antirabbit (1:10,000 dilution) for RhoA/ROCK II, MYPT1, p-MYPT1, and MLC20; bovine antibody (LI-COR Biosciences.) in dark [bovine antirabbit (1:10,000 dilution)] for p-MLC20]. After three more 10-min wash cycles in Tris-buffered saline-Tween 20, the membranes were kept in PBS on a shaker for 10 min at RT in dark and scanned using a LI-COR infrared scanner, and the integrated optical densities were determined using ImageJ software (National Institutes of Health, Bethesda, MD). The relative densities were calculated by normalization of the expression of each protein to that of α-actin.

ROCK activity measurement. ROCK activity was measured in IAS tissue homogenates under control and following treatment with LY-83583, before and after 10 and 100 μM of l-arginine according to the method published previously (45). The smooth muscle tissue strips were flash-frozen using Wollenberger clamps precooled in liquid nitrogen (34) and homogenized in ice-cold lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, a protease inhibitor mixture, and Na3VO4, a phosphatase inhibitor (Pierce). The protein concentration in tissue lysates was determined using a BCA protein assay kit (Pierce). Kinase activity was measured using a nonradioactive kinase assay kit (catalog no. STA-416; Cell Biolabs). An equal amount of protein lysates was used for each sample assayed in quadruplicate. ELISA was performed two times, and absorbance was measured at 450 nm according to the manufacturer’s instructions.

NO measurement. For this, we followed the previously established protocol by Ye et al. (55), with slight modifications. 4,5-Diaminofluorescein (DAF-2, 10 μM) solution was made in 0.1 M phosphate buffer (pH 7.4) in 50-μl aliquots in 96-well plates. Muscle bath perfusates (50 μl), before and after EFS, were quickly removed and mixed with DAF-2 solution. Normal KPS was used as negative control in these experiments. Fluorescence intensity was measured at absorbance and emission wavelengths of 490 and 510 nm, respectively, using a fluorescence plate reader.

Monitoring of oxidative stress. The oxidative stress measurement protocol was adopted from previously published studies (38) using DHE IFI determination. The IAS SMCs were grown on cover slips or in 96-well plates. For optimization, a batch of SMCs was treated with LY-83583 (10−9 M to 10−4 M) for different time periods. In a separate series of experiments, the SMCs were preloaded with DHE (5 μM) in DMEM for 30 min, and following addition of LY-83583 (10−5 M) these SMCs were incubated at 37°C for different time points (10 or 30 min), before and after 100 U/ml SOD pretreatment. To preserve the IFI signal, the cells were fixed with 4% paraformaldehyde, washed with PBS three times, mounted with Prolong Gold mounting medium, and covered with a cover slip. The slides were examined under the ultraviolet microscope and imaged using Texas Red filter. IFI (%maximal change) was measured using Image-Pro software, and graphs were plotted using Prism version 5.1.

Chemicals and drugs. Bovine erythrocyte SOD, LY-83583, DHE, DAF-2, l-NNa, SOD, l-arginine, and α-actin were obtained from Sigma (St. Louis, MO). U-46619 and Y-27632 were from Biomedical (Plymouth Meeting, PA). RhoA, ROCK II, α (Thr0690)-MYPT1, and p (Thr18/Ser19)-MLC20 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye680- and IRDye800-conjugated mouse, goat, and rabbit secondary antibodies were from LI-COR Biosciences.

Data analysis. Values were calculated as means ± SE from at least four independent experiments and plotted using Prism 5.1 (GraphPad Software, La Jolla, CA). Statistical significance was tested by one-way ANOVA followed by Dunnett’s post hoc test when three or more different groups were compared. To compare two different groups, the unpaired Student t-test was used. A P value <0.05 was considered to be statistically significant.

RESULTS

Effect of LY-83583 on basal IAS tone. The oxidative stress inducer LY-83583 produced a concentration (0.1 nM to 100 μM)-dependent bimodal effect in IAS tone. Lower concentrations (0.1 nM to 10 μM) produced concentration-dependent increases, whereas higher concentrations (>10 μM) produced precipitous decreases in the IAS tone (P < 0.05; n = 4; Fig. 1, A and B). Figure 1A shows a typical tracing of such experiments, and Fig. 1B shows the quantitative data from multiple

Fig. 2: A: typical tracing of an experiment to show that Y-27632 attenuates a maximal increase in the IAS tone caused by LY-83583. B: time-course effect of 10−5 M of LY-83583 before (control, green) or after either Rho kinase (ROCK) inhibitor Y-27632 (blue) or PKC inhibitor Gö-6850 (red) given after a sustained increase in IAS tone with LY-83583 (at 70 min of the progression of such experiments). Use of Ca2+-free (0Ca2+ KPS) or Y27632 (10−6 M) solution was made in 0.1 M phosphate buffer containing 0.1% Tween 20 for 1 h at RT. After three 10-min wash cycles in Tris-buffered saline-Tween 20, the membranes were incubated with the IRDye680- and IRDye800-conjugated secondary antibody (LI-COR Biosciences) in dark (bovine antirabbit (1:10,000 dilution) for RhoA/ROCK II, MYPT1, p-MYPT1, and MLC20) bovine antibody (LI-COR Biosciences) in dark (bovine antirabbit (1:10,000 dilution) for p-MLC20). After three more 10-min wash cycles in Tris-buffered saline-Tween 20, the membranes were kept in PBS on a shaker for 10 min at RT in dark and scanned using a LI-COR infrared scanner, and the integrated optical densities were determined using ImageJ software (National Institutes of Health, Bethesda, MD). The relative densities were calculated by normalization of the expression of each protein to that of α-actin.

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experiments. The effects of LY-83583 were reversible after washing.

Effect of ROCK inhibitor Y-27632 on the LY-83583-induced increase in IAS tone. To determine the effect of Y-27632 on the LY-83583-induced increase in the IAS tone, we performed two series of experiments.

In the first series, we first obtained a maximal effect with $10^{-5}$ M LY-83583, followed by the addition of different concentrations of Y-27632, as shown in a typical tracing in Fig. 2A. Quantitative data of such experiments are shown in the time-course protocol in Fig. 2B. Herein, we first established the actual basal IAS tone by replacement of normal KPS with KPS containing $0\text{Ca}^{2+}$, followed by the rest of the experiments in normal KPS. We compared the time-course effect of LY-83583 before and after ($10^{-6}$ M) Y-27632. Data showed that LY-83583 (added at 27 min of the progression of these experiments) produced a sustained increase in the IAS tone in control experiments; Y-27632 caused a significant decrease in the sustained increase ($P < 0.05; n = 4$). In contrast, the protein kinase C (PKC) inhibitor Gö-6850 ($10^{-6}$ M) had no significant effect on this sustained increase ($P > 0.05; n = 4$).

In the second series, we examined the effects of 10 µM LY-83583 before and after different concentrations of Y-27632 ($10^{-9}$ to $10^{-7}$ M) and $10^{-6}$ M Gö-6850 as shown in Fig. 2C. As shown, following the maximal increase in the IAS tone with LY-83583, Y-27632 produced concentration-dependent decreases in the IAS tone ($P < 0.05; n = 4$) while PKC inhibitor Gö-6850 had no significant effect ($P > 0.05$).

Altogether, these data suggest that the LY-83583-induced increase in IAS tone is mediated via RhoA/ROCK and not via PKC activation.

Concentration-dependent increase in oxidative stress by LY-83583: influence of SOD. In these experiments, we monitored changes in oxidative stress before and after LY-83583 using DHE staining of the IAS SMCs. Florescent microscopic images revealed a concentration-dependent increase in oxidative stress by LY-83583 (Fig. 3A). A maximal increase in oxidative stress was observed at 100 µM of LY-83583. Data further showed that an increase in oxidative stress was critically dependent on DHE incubation time. Thirty minutes of incubation produced a generalized increase in oxidative stress as reflected by a significant increase in IFI by all concentrations of LY-83583 ($P < 0.05; n = 4$; Fig. 3B). However, the increase in oxidative stress was more specific with the lower incubation time of 10 min, as revealed by the concentration specificity. Lower concentrations of LY-83583 ($10^{-9}$ to $10^{-7}$ M) produced no significant change, midrange concentrations ($10^{-6}$ to $10^{-5}$ M) produced a modest but significant increase ($P < 0.05; n = 4$), whereas higher concentrations ($10^{-5}$ M) led to a further increase in IFI ($P < 0.05; n = 4$). Importantly, as shown by data in Fig. 3B, changes in the IAS tone with LY-83583 correlate with those in oxidative stress. Moderate levels of oxidative stress produced a concentration-dependent increase while severe oxidative stress led to a precipitous decrease in IAS tone.

Interestingly, LY-83583 induced increase in oxidative stress (Fig. 3, A and C), and tone (Fig. 3D) was significantly atten-

Fig. 3. A: microscopic images with dihydroethedium (DHE) staining showing the effect of different concentrations of LY-83583 ($10^{-9}$ to $10^{-4}$ M), and the effect of superoxide dismutase (SOD, 100 U/ml) on LY-83583 ($10^{-3}$ and $10^{-4}$ M). B: correlation between % increase in immunofluorescence intensity (IFI) and changes in the IAS tone with different concentrations of LY-83583. Ten minutes (compared with 30 min) incubation time with DHE (in the presence of higher concentrations vs. $10^{-6}$ M of LY-83583) provides a more specific and significant increase ($*P < 0.05; n = 4$) in IFI that correlates with the changes in IAS tone. Data show that a further significant increase in IFI with $10^{-4}$ vs. $10^{-3}$ M of LY-83583 ($**P < 0.05; n = 4$) is associated with a decrease in IAS tone. In addition, SOD significantly ($*P < 0.05; n = 4$) and concentration-dependently attenuates LY-83583-induced increases in IFI (C) and the increase in IAS tone (D).
Control

These increases are reversible by Y-27632 (LY-83583 (compared with basal values). These increases are reversible by Y-27632 (LY-83583).

Effect of LY-83583 on IAS tone increased by U-46619 (stable analog of thromboxane A₂, and activator of RhoA/ROCK). To examine the role of RhoA/ROCK (5) in the U-46619-induced increase in IAS tone, and vice versa. In the first series of experiments, data showed that U-46619 in a concentration-dependent manner attenuated an increase in the IAS tone caused by LY-83583 (Fig. 4A). Given the role of RhoA/ROCK in both the excitatory effect of LY-83583 (Fig. 2) and the contractile effect of U-46619 in smooth muscle (13, 24, 31, 43, 50, 56), data suggest a competition between the actions of U-46619 and LY-83583 for producing an increase in the IAS tone. To further test this, the experiments performed in reverse showed a significant shift in the U-46619 CRC toward the right following pretreatment with LY-83583 (P < 0.05; Fig. 4B). However, in contrast, a decrease in the IAS tone by higher concentrations of LY-83583 was not affected by U-46619 (P > 0.05; n = 4; Fig. 4C). Altogether, these data suggest the role of RhoA/ROCK in the LY-83583-induced increase in IAS tone (P < 0.05; Fig. 4B).

Effect of LY-83583 on RhoA/ROCK activation. To further determine the role of RhoA/ROCK activation, we investigated the effect of LY-83583 on RhoA/ROCK translocation. Data revealed that in the lower concentrations LY-83583 caused a significant increase in Rhoa (P < 0.05; n = 4; Fig. 5A) and ROCKII (P < 0.05; n = 4; Fig. 5B) in the particulate fractions, which were attenuated by Y-27632 (10⁻⁶ M). Interestingly, as
shown before (43), higher concentrations of LY-83583 decreased the levels of RhoA/ROCK in the particulate fractions. The role of RhoA/ROCK in the effects of LY-83583 was further confirmed by ROCK activity assays. These data showed a significant \( (P < 0.05; n = 4); \text{Fig. 5C} \) and concentration-dependent increase in ROCK activity with lower concentrations of LY-83583 \((10^{-7} \text{ to } 10^{-5} \text{ M})\) and a decrease with higher concentration \((10^{-4} \text{ M})\) \((P < 0.05); \text{Fig. 5C} \).

**Effect of oxidative stress on p\(^{\text{Thr90}}\)-MYPT1 and p\(^{\text{Thr180Ser190}}\)-MLC\(_{20}\).** Lower concentrations of LY-83583 significantly increased levels of p-MYPT1 and p-MLC\(_{20}\) \((P < 0.05; n = 4); \text{Fig. 6, A and B} \) in the IAS smooth muscle strips. These increases caused by \(10^{-5} \text{ M} \) LY-83583 were significantly attenuated by \(10^{-6} \text{ M} \) Y-27632 \((P < 0.05; n = 4); \text{Fig. 6, A and B} \).

**Effect of NOS inhibitor L-NNA on basal IAS tone: influence of ROCK inhibitor.** L-NNA \((100 \text{ µM})\), similar to LY-83583, produced a significant increase in IAS tone \((P < 0.05; n = 5); \text{Fig. 7, A and B} \) that was significantly attenuated by Y-27632 in a concentration-dependent manner \((P < 0.05; n = 4); \text{Fig. 7B} \). A typical tracing of such experiments is shown in Fig. 7A.

**Effect of NOS inhibition.** LY-83583 \((10 \text{ µM})\) significantly attenuated EFS-induced relaxation in the IAS. L-Arginine (and not D-arginine) and SOD caused concentration-dependent reversal of this attenuation \((P < 0.05; n = 5–8); \text{Fig. 8, A and B} \). Typical tracings of such experiments are given in Fig. 8, A and B, bottom.

As evidence for the role of NOS inhibition in the effect of LY-83583, we measured NO levels from organ bath perfusates of the IAS smooth muscle strips in response to EFS, before and after LY-83583. As shown in Fig. 9, EFS caused a frequency-dependent increase in NO release, and LY-83583 significantly inhibited such release \((P < 0.05; n = 5–8)\), which was selectively reversed by L-arginine \((10^{-4} \text{ M})\) to the levels not significantly different from control values \((P > 0.05)\).

**DISCUSSION**

These studies, for the first time, demonstrate a bimodal effect of oxidative stress in basal tone and the underlying control mechanisms in intact IAS. The present findings suggest: 1) a lower level of oxidative stress leads to an increase while higher levels cause a decrease in IAS tone; 2) mechanistically, these changes in the IAS are associated with changes in RhoA/ROCK; and 3) Rho/ROCK activation responsible for the increase in IAS tone by lower levels of oxidative stress may in part be associated with neuronal nitric oxide synthase (nNOS) inhibition.

In these studies, we used a wide range of LY-83583 concentrations to simulate different levels of oxidative stress \((4, 30)\), which was monitored using DHE staining IFI \((38, 43)\) of

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**Fig. 6.** Western blot analysis showing significant increase in phosphorylated (p)-myosin phosphatase target subunit 1 (MYPT1, A) and p-20 kDa myosin light chain (MLC\(_{20}\), B) \((^*P < 0.05; n = 4)\) following LY-83583 \((10^{-7} \text{ to } 10^{-5} \text{ M})\) pretreatment. These increases caused by LY-83583 \((10^{-7} \text{ M})\) are significantly \((**P < 0.05; n = 4)\) attenuated by Y-27632 \((10^{-6} \text{ M})\) (LY-5/Y2-6).

**Fig. 7.** Typical tracing (A) and quantitative data (B) showing that Y-27632 in a concentration \((10^{-9} \text{ to } 10^{-6} \text{ M})\)-dependent manner causes significant \((^*P < 0.05; n = 4)\) attenuation of an increase in IAS tone caused by L-NNA \((100 \text{ µM})\).
the SMCs. Specific experiments in this regard showed that LY-83583 causes a concentration-dependent increase in oxidative stress that correlates with the changes in the IAS tone. LY-83583 (up to $10^{-5}$ M) produces a low grade or moderate increase while higher concentrations produce a severe increase in oxidative stress as determined by DHE IFI.

Data show that an increase in the IAS tone occurs primarily via RhoA/ROCK activation and does not involve PKC activation. This is in agreement with earlier studies from different laboratories showing that RhoA/ROCK rather than PKC activation is primarily responsible for the sphincteric tone in animals and humans (29, 32, 34, 37, 41). On the other hand, a decrease in IAS tone by higher levels of oxidative stress (simulated by LY-83583 in concentrations higher than $10^{-5}$ M) occurs via inactivation of RhoA/ROCK (43).

Evidence for the role of RhoA/ROCK activation for increase in the basal IAS tone (simulated by the lower concentrations of LY-83583) emerges from the following series of data. ROCK inactivation by Y-27632 in a concentration-dependent manner restores the elevated tone. This effect of Y-27632 was observed in the lower concentrations, previously demonstrated to be selective for ROCK inactivation (35). Additionally, the PKC inhibitor Gö-6850 fails to modify the effect of LY-83583. Data further show that lower concentrations of LY-83583 translocate RhoA/ROCKII to the membrane, which is significantly attenuated by Y-27632. Additionally, direct measurements of ROCK activity, and signal transduction changes in p-MYPT1 and p-MLC$_{20}$ before and after Y-27632, provide further evidence for the role of RhoA/ROCK in the increase in IAS tone by lower concentrations of LY-83583.

The LY-83583-induced increase in IAS tone closely resembles that of the increase by U-46619 (shown previously), since both are attenuated by Y-27362. In addition, U-46619 and LY-83583 attenuate each other’s CRC, showing an increase in IAS tone. These data further support the role of RhoA/ROCK in response to lower levels of oxidative stress, since U-46619

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**Fig. 8.** Quantitative data for these experiments showing significant (*$P < 0.05$; $n = 5–8$) attenuation of the electrical field stimulation (EFS) frequency-response curves by LY-83583 and its reversal (**$P < 0.05$; $n = 5–8$) by l-arginine (but not by d-arginine; $P > 0.05$) (A) and SOD (B). The quantitative data on top are illustrated in typical tracings of such experiments in the bottom panels of A and B.

**Fig. 9.** EFS causes a frequency-dependent increase in NO release in the IAS smooth muscle perfusates. This increase in NO release is attenuated following pretreatment of the smooth muscle strips with LY-83583 (*$P < 0.05$; $n = 5–8$), and this attenuation is reversible l-arginine to the levels that are not significantly different from control values.

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is a well-known activator of RhoA/ROCK for smooth muscle contractility (54). These data are interpreted to mean that both of these agents compete for the same pathway in increasing the IAS tone.

The present data with the lower concentrations of LY-83583 in intact IAS reveal a striking resemblance with nNOS inhibition by L-NNA: an increase in tone and attenuation of EFS-induced relaxation. The increase in IAS tone is attenuated by ROCK inhibitor Y-27632, and EFS responses are selectively reversible by L-arginine and SOD. Data with EFS before and after LY-83583, monitoring direct release of NO, further reveal the involvement of nNOS in IAS relaxation. The role of nNOS in the nonadrenergic, noncholinergic relaxation of IAS by EFS and rectoanal inhibitory reflex has been demonstrated previously (33, 36).

The exact significance of parallels between the effects of LY-83583 and nNOS inhibition, however, is not clear at the present time. Speculatively, lower levels of oxidative stress via NOS inhibition may in part provide intramural signal for RhoA/ROCK activation. Interestingly, it has been shown previously that nNOS inhibition produces a tissue- and species-specific increase in IAS tone during pharmacological and pathophysiological conditions (26, 48, 51).

It is also possible that the increase in IAS tone via inhibition of NOS, guanylyl cyclase, or by ONOO– anion may activate RhoA/ROCK pathway (18, 20, 47). In independent studies, we have observed that ONOO– increases the IAS tone (data not shown). The basal state of smooth muscle contractility is considered to be a result of net balance between multiple intracellular excitatory and inhibitory pathways. Therefore, nNOS activation (as a consequence of severe oxidative stress) or inhibition (by moderate oxidative stress) may play an important role for the eventual changes in basal IAS tone. Pathophysiology and therapeutic targeting in relation to oxidative stress may be of relevance in RI (associated with hypertensive IAS) and recurrent anal fissure (often associated with hypertensive IAS) (7).

Whether lower levels of oxidative stress are involved in rectoanal motility disorders characterized by hypertensive IAS such as in Hirschsprung’s disease (HPD), recurrent anal fissure (RAF), and hemorrhoids (16, 23, 25), remains to be determined. Hirschsprung’s disease has been associated with a decrease in nNOS myenteric neurons (28). One of the therapeutic approaches for such disorders has been NO-releasing substances (1) or PDE V inhibitors, expected to maximize the neuromuscular effect following compromised release of NO in such conditions (49). An additional rational therapy of hypertensive IAS smooth muscle may be RhoA/ROCK inhibitor such as Y-27632 (32, 34). If established, these data would further suggest a link between nNOS inhibition, lower levels of oxidative stress, and an overexpression of the RhoA/ROCK pathway.

In contrast to the effect of lower levels of oxidative stress, higher levels cause a decrease in IAS tone associated with a decrease in RhoA/ROCK activity, as shown here and in an earlier publication from our laboratory (43). Such data may have relevance in the pathophysiology and therapeutic targeting of RI during advanced aging, associated with severe changes in oxidative stress (8, 15, 15, 43, 53).

In summary, the present data suggest that oxidative stress has a bimodal effect in IAS tone. In lower levels it increases while at higher levels it decreases the IAS tone. Whether these levels are associated with the clinical hypertensive IAS and hypertensive IAS, respectively, remains to be determined. (It is well known that hypertensive IAS is associated with certain rectoanal motility disorders such as HPD, RAF, and hemorrhoids. Hypotensive IAS, on the other hand, has been associated with certain forms of RI.) A determination of such association is expected to advance our understanding of the pathophysiology and therapeutic targeting of the above rectoanal motility disorders. In this regard, antioxidants such as SOD, in appropriate concentrations may have an important role in reversing these disorders and aging-associated oxidative damage in IAS.

REFERENCES


