Reactive oxygen species are messengers in maintenance of human and guinea pig gallbladder tonic contraction

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Cong P, Xiao Z-L, Biancani P, Behar J. Reactive oxygen species are messengers in maintenance of human and guinea pig gallbladder tonic contraction. Am J Physiol Gastrointest Liver Physiol 293: G1244–G1251, 2007.—The tonic contraction of human and guinea pig gallbladder (GB) is dependent on basal levels of PGE_2 and thromboxane A_2 (TxA_2). The pathway involved in the genesis of these prostaglandins has not been elucidated. We aimed to examine the source of reactive oxygen species (ROS) and whether they contribute to the genesis of GB tonic contraction by generating basal prostaglandin levels. Tonic contraction was studied in human and guinea pig GB muscle strips treated with ROS scavengers (Tiron and catalase), apocynin (an inhibitor of NADPH oxidase), and NOX-1 small interference RNA (siRNA). The subunits of NADPH oxidase and their functional roles were determined with specific antibodies in GB muscle cells. ROS scavengers reduced the tonic contraction and H_2O_2 and PGE_2 levels. Apocynin also inhibited the tonic contraction. Antibodies against subunits of NADPH oxidase present in GB muscle cells lowered H_2O_2 and PGE_2 levels. NOX-1 siRNA transfection reduced the tonic contraction, NOX-1 expression, and levels of H_2O_2 and PGE_2. Tiron and apocynin inhibited the expected increase in tension and H_2O_2 levels induced by stretching of muscle strips. H_2O_2 increased the levels of PGE_2 and TxA_2 by increasing platelet-activating factor-like lipids that phosphorylate p38 and cPLA_2 sequentially. H_2O_2 generated by NADPH oxidase participates in a signal transduction pathway that maintains the GB tonic contraction by activating PAF, p38, and cPLA_2 to generate prostaglandins.

Muscle; NADPH oxidase; prostaglandins

We have recently shown that gallbladder (GB) tonic contraction is dependent on basal levels of thromboxane A_2 (TxA_2) and prostaglandin E_2 (PGE_2) generated by the activity of constitutive cyclooxygenase (COX)-1 and COX-2 enzymes, respectively (9). Both prostaglandins act on specific G protein-coupled receptors, thromboxane prostanoid receptors of TxA_2 and E prostaglandin (EP-1) receptors of PGE_2 (5, 19, 32, 33). These prostaglandin receptors couple to Gq/11 proteins causing a sustained contraction by acting on PKC (4). However, the signaling pathways that stimulate the basal activity of COX enzymes to generate prostaglandins that contribute to the maintenance of tonic tension have not been elucidated.

We have also shown that exogenous and endogenous reactive oxygen species (ROS) stimulate cytotoxic protective mechanisms by increasing PGE_2 levels that activate catalase, a free radical scavenger, and cause GB muscle contraction (39, 40). These findings are in agreement with previous studies that showed free radicals contracting a variety of smooth muscle cells in blood vessels, pulmonary tract, and gallbladder muscle cell by stimulating the synthesis of prostaglandins or by interacting with nitric oxide (14, 16, 24, 29, 31, 37, 38).

The aim of the present studies was to determine whether ROS (H_2O_2) also behave as signaling molecules that contribute to GB tonic contraction by activating pathways that stimulate the synthesis prostaglandins.

METHODS

Patients. Human GBs were obtained by elective laparoscopic cholecystectomy performed for gallstone disease. Muscle strips and cells were obtained from human GB with pigment stones determined by their gross appearance (black stones) and chemical analysis (1, 3, 11). None of the patients had clinical or pathological evidence of acute cholecystitis. We have previously shown that human GBs with pigment stones contract normally in response to a constant intravenous infusion of CCK-8 since the GB ejection fraction is not different from than of normal subjects without gallstones (1). The muscle strips from these GBs exhibit a normal tonic contraction (3) and contract normally to a variety of agonists such as CCK-8, ACh, PGE_2, and KCl that are not different from the responses of GB muscle cells from a variety of normal animal species (11, 41, 42).

These muscle cells also are not exposed to abnormal levels of oxidative stress since the levels of H_2O_2, lipid peroxidation, PAF, and PGE_2 and the activity of catalase, a free radical scavenger, are not different from those found in GB muscle cells from normal guinea pigs (Table 1) (39, 40).

Animals. Male guinea pigs (weight 450–500 g) were purchased from the Charles River Laboratory (Wilmington, MA). The Animal Welfare Committee of Rhode Island Hospital has approved the protocol. Animals were housed in thermoregulated rooms with free access to food and water. After an overnight fast, the guinea pigs were sedated with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and euthanized by pentobarbital (30 mg/kg ip).

Human and guinea pig GB were promptly removed, rinsed with ice-cold oxygenated Krebs solution, and placed in a dissecting pan containing the same solution continuously aerated with 95% O_2-5% CO_2. The GB was kept in ice-cold oxygenated Krebs solution (116.6 mM NaCl, 3.4 mM KCl, 21.9 mM NaHCO_3, 1.2 mM NaH_2PO_4, 2.5 mM CaCl_2, 1.2 mM MgCl_2, 5.4 mM glucose). The mucosa and serosa were carefully peeled off under a dissecting microscope. The GB muscle layer was further cleaned by gently removing the remaining connective tissue.

Isolation and permeabilization of GB muscle cells. Muscle cells were isolated and in some experiments they were permeabilized by methods described previously (32, 33, 36, 37). The muscle layer was cut into 2-mm-wide strips and digested in HEPES buffer containing 0.5 mg/ml type F collagenase and 2 mg/ml papain (activity of 13.9

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Table 1. Basal tonic contraction and parameters of oxidative stress in GB muscle strips from human and guinea pigs

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Tonic Contraction</th>
<th>H2O2</th>
<th>PGE2</th>
<th>TxB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>9</td>
<td>1.97±0.54*</td>
<td>2.39±0.1</td>
<td>6.87±1.5</td>
<td>4.12±0.8</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>21</td>
<td>1.49±0.39</td>
<td>2.2±0.13</td>
<td>6.8±2</td>
<td>4.2±0.6</td>
</tr>
</tbody>
</table>

Value are means ± SE, *P = 0.47. GB, gallbladder; TxB2, thromboxane B2.

U/mg protein) for 20 min at 35°C in a shaking water bath. The buffer was gently gassed with 100% O2 during the tissue digestion. At the end of the digestive process, the tissue was filtered through Nitex mesh 200 (Tetko, Elmsford, NY) and rinsed with 20 ml of HEPES. The tissue remaining on the filter was collected and incubated in HEPES buffer at 35°C for 15 min to allow free dispersion of cells.

For preparation of permeable cells, the partly digested muscle cells were washed with “cytosolic buffer,” a medium with composed of (in mM) 20 NaCl, 100 KCl, 25 NaHCO3, 0.96 NaH2PO4, 0.48 CaCl2, 5.0 MgSO4, and 1.0 EGTA, pH 7.2, 2% bovine serum albumin, and was maintained by equilibration with 95% O2-5% CO2 at 31°C. The dispersed cells were exposed to saponin (75 µg/ml) and then centrifuged at 200 g for 3 min. Cells were washed once with modified cytosolic buffer by centrifugation and resuspended in modified cytosolic buffer and equilibrated at 31°C for 15 min before the experiment. The modified cytosolic buffer was prepared with cytosolic buffer plus 1.5 mM ATP, 5 mM creatine phosphate, 10 U/ml of creatine phosphokinase, and 10 µM antimycin A.

Studies on muscle cell contraction. Muscle contraction was measured as previously described (1, 9, 39, 40). Permeable cells were pretreated with buffer (control) or antibodies against p22phox, p47phox, p67phox, and Rac-1, exposed the cells to 50 mM taurochenodeoxycholate acid (TCDC), and then fixed in acrolein at 1% final concentration. The cell length was measured with a phase-contrast microscope (Carl Zeiss, Jena, Germany) and a closed-circuit television camera (Panasonic, Secaucus, NJ) connected to a Macintosh computer with NIH Image software. The average length of 30 cells, measured in the absence of agonists, was taken as the “control” length and compared with length measured after addition of agonists. Shortening was defined as the percent decrease in the average length of 30 cells after treatment with agonists compared with the control length.

siRNA transfection of human muscle strips in organ culture. Organ culture of muscle strips and small interference RNA (siRNA) transfection were performed by the method described before (9, 26). Briefly, human GB muscle strips devoid of mucosa and serosa were rinsed several times with sterile Krebs buffer and then placed in serum and antibiotic free RPMI 1640 medium containing scramble or NOX-1 siRNA (100 pmol) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. After a 24-h transfection, muscle strips were rinsed in warm PBS and placed into a muscle bath for further studies to determine the active tonic contraction as described below. All muscle strips treated with sense (5'-TTAACAGACGCCGCTCCTG-3') and anti-sense (5'-CTGGAGAAATGGAGCCCCAG-3') siRNA were examined for the expression of the target protein by Western blotting. Transfection experiments were performed in triplicate. Data represent the mean of three different GB specimens.

Transfection efficiency studies. The efficiency of the siRNA transfection was verified with a fluorescent-labeled oligo (Invitrogen) that was transfected to the muscle strip at the same concentration of the transfection mixture (100 pmol/ml) and vector lipofectamine. After 24 h incubation, muscle strips were frozen and tissue sections (10 µM) were placed on slides. The optimal transfection efficiency obtained via an Olympus IX50/FIA fluorescent microscope equipped with a nap-fix camera (Olympus Optical, Melville, NY) was 65%.

Measurement of tonic contraction in muscle strips. Strips were mounted in 1-ml muscle chambers as described in detail previously (3, 4, 10). Briefly, muscle strips were initially stretched to 2.5 g of passive force and equilibrated by continuous perfusion with oxygenated Krebs solution at 37°C. After 1-h perfusion, a basal spontaneous contraction gradually developed and stabilized after another 30-min period of equilibration. Stable tonic contraction of control and treated muscle strips was measured with Grass isometric force transducers and

Fig. 1. A: tracings illustrate the inhibitory effects of Tiron (10^-5 M), a cell-permeable free radical scavenger, on tonic contraction of human and guinea pig gallbladder (GB) muscle strips. B: effects of increasing concentrations of Tiron (10^-5 to 10^-4 M) on tonic contraction in muscle strips from guinea pigs compared with control strips. Values are means ± SE of n = 3 (P < 0.01 per ANOVA). C: effect of Tiron (10^-3 M) on H2O2 and PGE2 levels in human GB muscle strips (*P < 0.01). Tiron lowered H2O2 and PGE2 levels. Values are means ± SE of n = 3 (**P < 0.001, by unpaired Student’s t-test).
amplifiers connected to a Biopac data-acquisition system. Active tension was the difference between total minus passive tension obtained after 20 mmol/l EGTA added at the end of the experiment (9). In separate experiments, the effect of gradual muscle stretch on tonic contraction and H₂O₂ production were examined by stretching the strips by 1, 2, 3, or 4 g 1 h before tonic contraction or biochemical studies were performed (9).

Western blot. Muscle strips were homogenized in Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (vol/vol) Triton X-100, 40 mM β-glycerophosphate, 40 mM p-nitrophenylphosphate, 200 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 µg/ml aprotonin. The suspension was centrifuged at 15,000 g for 5 min, and the protein concentration in the supernatant was determined. Western blot was done as previously described (9). Briefly, these supernatants were subjected to SDS-PAGE and the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V overnight. The nitrocellulose membranes are blocked in 5% nonfat dry milk and then incubated with anti-phosphorylated P38 MAPK antibody (1:5,000) or anti-phosphorylated cPLA₂ antibody (1:1,000) for 1 h (P38MAPK) or overnight (cPLA₂), followed by 60-min incubation in horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.6 mM Tris-HCl, pH 6.7) at 50°C for 30 min, washed three times (10 min each), and then reprobed by using anti-P38 antibody (1:500) or anti-cPLA₂ antibody (1:1,000), respectively.

Measurements of H₂O₂ levels. The H₂O₂ content was determined as previously described by using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) (Amplex, Molecular Probes, Eugene, OR) (37). It uses the Amplex Red reagent (10-acetyl-3, 7-dihydroxyphenoxazines) to detect H₂O₂, spectrophotometrically. In the presence of peroxidase, the Amplex Red reagent reacts with H₂O₂ in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. The content of H₂O₂ was calculated from the standard curve and expressed as micromoles per liter per milligram of protein.

**Results.** The differences between controls and experimental results were determined by ANOVA or unpaired Student’s t-tests.

**RESULTS**

The active tension of human GB muscle strips was 1.97 ± 0.54 g (n = 9 specimens), slightly higher than the tension of guinea pig GB muscle strips of 1.49 ± 0.39 g (n = 21 specimens, P = 0.47, Table 1). However, there were no significant differences in the parameters of oxidative stress between human and guinea pig GB muscle strips. The H₂O₂ levels were 2.39 ± 0.1 µM/mg proteins for human compared with 2.2 ± 0.1 µM/mg protein for guinea pig GB; PGE₂ levels
ROS MESSENGERS IN MAINTENANCE OF HUMAN AND GUINEA PIG GALLBLADDER TONIC CONTRACTION

Effect of scavengers of ROS (Tiron and catalase) on GB tonic contraction. Muscle strips develop a steady tonic contraction (Fig. 1A). Tiron (10^{-5}M) (a cell-permeable scavenger of free radicals) decreased the active tonic contraction. Increasing concentrations of Tiron (10^{-5} to 10^{-4} M) showed a dose-dependent decrease in the guinea pig GB tension (Fig. 1B, P < 0.01, ANOVA). The maximal concentration reduced the active tension from 1.7 ± 0.3 to 0.5 ± 0.1 g in human GB muscle strips (Fig. 1B). Tiron also lowered the H_{2}O_{2} levels from 2.2 ± 0.2 to 0.77 ± 0.1 μM/mg protein (Fig. 1C, P < 0.01) and the PGE2 levels (P < 0.01, Fig. 1C). Likewise, catalase (the cell-impermeable scavenger of free radicals) also reduced the human and guinea pig GB tonic contraction (Fig. 2, A and B, P < 0.05, n = 4), suggesting that the final molecular form of the ROS that contribute to the tonic contraction is H_{2}O_{2} and that this ROS is released into the extracellular space since catalase is not cell permeable.

Cytosolic NADPH oxidase as the source of H_{2}O_{2}. We next examined whether NADPH oxidase is the source of ROS that contribute to the maintenance of GB tonic contraction. Muscle strips were treated with apocynin (16), an inhibitor of NADPH oxidase. Apocynin blocks the recruitment of p47phox and p67phox to the plasma membrane to activate the other subunits of this enzyme (28).

Fig. 3. A: Western blots demonstrating the presence of 5 subunits of the cytosolic NADPH oxidase (Nox-1, RAC-1, p22, p47phox, and p67phox) in muscle cells from human and guinea pig GB. B: effect of antibodies against NADPH oxidase subunits on the contraction induced by the hydrophobic bile acid taurocholaenoxycholate acid (TCDC). Each subunit blocked the contraction induced by TCDC (*P < 0.001, by unpaired Student’s t-test). However, these antibodies had no effect on CCK-8-induced contraction. Values are means ± SE of n = 3.

Fig. 4. Effect of antibodies against NADPH oxidase subunits p47, p67, and RAC-1 on basal H_{2}O_{2} (A) and PGE2 (C) levels in human GB muscle cells. These antibodies inhibited the production of H_{2}O_{2} and PGE2 (**P < 0.01). B and C: TCDC increased the levels of both H_{2}O_{2} and PGE2 levels (**P < 0.001, by unpaired Student’s t-test). Values are means ± SE of n = 3.

Fig. 5. Effect of NOX-1 small interference RNA (siRNA) on NOX-1 expression in human GB muscle strips compared with scramble siRNA or culture media alone. NOX-1 siRNA decreased significantly the expression of NOX-1 (*P < 0.001 by unpaired Student’s t-test). Values are means ± SE of n = 3.

of 6.87 ± 1.5 for human and 6.80 ± 2 nM/mg proteins for guinea pig GB; and TxB2 levels of 4.1 ± 0.8 in human and 4.2 ± 0.6 nM/mg protein in guinea pig GB muscle strips.

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Treatment of human and guinea pig GB muscle strips with apocynin (10^{-5} M) caused a gradual decline in GB tonic contraction (Fig. 2C) and decreased the tonic contraction mean values (Fig. 2D, P < 0.01), suggesting that this cytosolic enzyme is the source of ROS.

Moreover, the presence of NADPH oxidase in GB muscle cells was demonstrated by Western blot in human and guinea pig GB muscle cells using specific antibodies against subunits of the enzyme (Fig. 3A). These specific antibodies demonstrated the presence of NOX-1, RAC-1 (but not RAC-2), p22phox, p47phox, and p67phox in GB muscle cells from both species.

Furthermore, these NADPH oxidase subunits contribute to the generation of H2O2 and mediate GB muscle contraction (37). Treatment with specific antibodies against p22 phox, p47 phox, p67 phox, and RAC1 blocked the cell contraction induced by taurochenodeoxycholic acid, a hydrophobic bile acid (Fig. 3B). This bile acid induces GB muscle cell contraction by increasing H2O2 (38) and PGE2 levels (Fig. 4, B and C). Antibodies against p22phox, p47phox, p67phox, and RAC1 also lowered basal H2O2 and PGE2 levels (Fig. 4, A and C). These antibodies against NADPH subunits, however, had no effect on the CCK-8-induced contraction since it is not mediated by free radicals.

To further examine the role of NADPH oxidase and ROS in the maintenance of GB tonic contraction, muscle strips from human GB were transfected with NOX-1 siRNA for 24 h in tissue culture. The tonic contraction transfected with NOX-1 siRNA was compared with control muscle strips treated with tissue culture media alone or with scramble siRNA. NOX-1 siRNA decreased NOX-1 protein expression (Fig. 5, P < 0.01) and reduced the tonic contraction (Fig. 6A, P < 0.001) and PGE2 and H2O2 levels (Fig. 6, B and C, P < 0.01) compared with strips treated with tissue culture media or scramble siRNA. The muscle response to ACh (10^{-6} M) was used to test the viability of the muscle strips. The delta contraction in response to ACh was not different among all three treatments (9). The delta contraction in response to ACh after buffer or scramble siRNA treatment was 1.3 ± 0.4 g and after NOX-1 siRNA treatment was 1.26 ± 0.2 g (P > 0.05, n = 3).

We then examined whether increases in active tonic contraction induced by stretching GB muscle strips increase H2O2 levels. Passive stretching of human GB muscle strips resulted in increases in active tension that was length dependent (9). Gradual stretching of muscle strips to 1, 2, 3, and 4 g also caused a concomitant rise in the levels of H2O2 (P < 0.01, ANOVA). Pretreatment with Tiron (10^{-5} M) or with apocynin (10^{-5} M) prior to stretching the muscle strips inhibited increases in tonic contraction and in the levels of H2O2 (Fig. 7, A and B, P < 0.001, ANOVA). These studies suggest a cause
GB tonic contraction.

studied by using maximal concentrations H2O2 (70 μM) for 30 s before they were stimulated with H2O2 for 15 min. First we showed that H2O2 stimulates the synthesis of TxB2 (a stable TxA2 analog) and PGE2 that are known to generate GB muscle tonic contraction (Fig. 8, A and B) since TxA2 and PGE2 inhibit it receptor antagonists and by the nonselective (indomethacin) and selective COX antagonists (9). Their basal levels were reduced by treatment with antibodies against some of the NADPH oxidase subunits P22, p47phox, p67phox, and RAC-1 (Fig. 4, P < 0.01). The increases in the levels of prostaglandins was blocked by SB203580 a p38 inhibitor (10−5 M) and by AACOCF3 (3 × 10−5 M) a cPLA2 inhibitor (P < 0.001, Fig. 8).

H2O2 (70 μM) also increased p38 and cPLA2 phosphorylation that was blocked by PAF receptor antagonist CV-3988 but was unaffected by the inhibitor of cPLA2 (AACOCF3) or by the nonselective COX blocker indomethacin (Fig. 9A). H2O2 (70 μM) increased cPLA2 phosphorylation and was inhibited by the PAF receptor antagonist and the p38 inhibitor SB203580 but was unaffected by the nonselective COX inhibitor indomethacin (Fig. 9B). These findings suggest that both H2O2 and PAF utilize the same pathway to increase prostaglandin levels. They also suggest that the pathway sequence utilized by H2O2, and PAF is P38 that activates cPLA2 since p38 inhibitor (SB203580) blocked H2O2 and PAF induced cPLA2 phosphorylation. In contrast, the cPLA2 inhibitor (AACOCF3) did not block p38 phosphorylation (Fig. 9A). Moreover, indomethacin had no effect on p38 or cPLA2 phosphorylation.

**DISCUSSION**

The present studies show that H2O2, generated by the cytosolic enzyme NADPH oxidase, acts as a signaling molecule in a pathway involved in the maintenance of human and guinea pig GB tonic contraction. H2O2 is integrated in this pathway consisting of PAF-like lipids (15), p38 and cPLA2, that stimulates the synthesis of TxA2 and PGE2 (14, 20, 23, 25). These conclusions are supported by the findings that Tiron, a cell-permeable scavenger, and catalase, a cell-impermeable scavenger of free radicals, inhibited the human and guinea pig GB tension by lowering basal levels of H2O2.

The source of these ROS that participate in the tonic GB tension appears to be the cytosolic enzyme NADPH oxidase.

Muscle cells were pretreated with specific antagonists for 30 s before they were stimulated with H2O2 for 15 min. First we showed that H2O2 stimulates the synthesis of TxB2 (a stable TxA2 analog) and PGE2 that are known to generate GB muscle tonic contraction (Fig. 8, A and B) since TxA2 and PGE2 inhibit it receptor antagonists and by the nonselective (indomethacin) and selective COX antagonists (9). Their basal levels were reduced by treatment with antibodies against some of the NADPH oxidase subunits P22, p47phox, p67phox, and RAC-1 (Fig. 4, P < 0.01). The increases in the levels of prostaglandins was blocked by SB203580 a p38 inhibitor (10−5 M) and by AACOCF3 (3 × 10−5 M) a cPLA2 inhibitor (P < 0.001, Fig. 8).

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since apocynin, a NADPH oxidase blocker (21, 37), also inhibits the GB tonic contraction and lowers H2O2 levels. Apocynin inhibits NADPH oxidase by blocking the translocation of its subunits to the plasma membrane to generate the active enzyme (34). Moreover, identical subunits of NADPH oxidase seem to be present in both human and guinea pig GB muscle cells. Both have identical isoforms that include NOX-1, p22, p47phox, p67phox, and RAC-1 but not RAC-2. This enzyme has been shown to generate H2O2 since it mediates the action of compounds known to contract muscle cells by increasing ROS levels such as hydrophobic bile acids and endothelin (34, 38). In addition, specific antibodies against these subunits lowered basal H2O2 levels and blocked the contraction induced by hydrophobic bile acids that contract GB muscle cells by increasing H2O2 levels (38). Moreover, the finding that catalase lowers the GB tonic tension suggests that H2O2 is the final ROS. H2O2 is released into the extracellular space and stimulates the pathway that generates the GB tonic contraction by causing lipid peroxidation and increasing the synthesis of PAF and prostaglandins, particularly PGE2 (37, 39, 40).

These conclusions are also supported by the findings that transfection of muscle cells with NOX-1 siRNA reduced significantly the tonic contraction of human muscle strips compared with strips treated with culture media or transfected with scramble siRNA. The reduction of GB tension induced NOX-1 siRNA was associated with a lower NOX-1 protein expression and basal H2O2 levels.

The involvement of H2O2 in the genesis of GB tonic contraction was further demonstrated by the concomitant increase in tonic contraction and H2O2 levels induced by a gradual passive stretching of GB muscle strips. Moreover, both the gradual increase in active tension and H2O2 levels was blocked by pretreatment of the muscle strips with Tiron or apocynin. These findings are in agreement with previous studies showing that mechanical stretching of muscle strips leads to an increase in active tension and levels of TxA2 and PGE2 (9, 27). These studies also show some of the steps involved in the signal transduction that mediate H2O2-induced generation of TxA2 and PGE2. We have previously shown that H2O2 by inducing lipid peroxidation generates PAF-like lipids that act on specific G protein-coupled receptors since a specific PAF receptor antagonist blocked their actions (15). We now show that the pathway activated by H2O2 and PAF includes phosphorylation of p38 and cPLA2 that is known to release AA (12, 17, 29). H2O2 has also been reported to activate ERK 1/2 to activate pathways that leads to apoptosis and other pathways in a variety of tissues (2, 23, 25). H2O2, after generating TxA2 and PGE2, also stimulates ERK1/2 in GB muscle cells (J. Behar, unpublished observations). We have also shown that AA is hydrolyzed by COX-1 to form TxA2 and by COX-2 to generate PGE2 (9).

H2O2 and PAF contract GB muscle cells by stimulating prostaglandins since indomethacin, the non-specific COX inhibitor, blocked the contraction induced by either compound (15, 37). These prostaglandins by also acting on G protein coupled receptors contribute to the maintenance of GB contraction since specific prostaglandin receptor antagonists decrease the tonic contraction of human and guinea pig GB muscle strips (9).

It has also been shown that ROS could also cause contraction of certain muscle cells by inactivating nitric oxide. However, nitric oxide does not play a role in the relaxation of GB muscle strips since inhibitors of NOS do not change GB tonic contraction or blocked GB relaxation induced by electrical field stimulation (12).

In contrast to the low intracellular H2O2 concentrations that appear to function as signaling molecules, oxidative stress and inflammatory processes generate higher concentrations that damage plasma membrane receptors and deplete calcium stores resulting in reductions in lower esophageal sphincter and GB tonic contraction (6, 12, 43) and in colonic phasic motor activity (7, 8).

In summary, the present studies showed that basal levels of ROS (H2O2) function as a second messenger in a pathway that participates in the maintenance of human and guinea pig GB tension. It is generated by the cytosolic enzyme NADPH oxidase and by producing PAF-like lipids stimulates p38, cPLA2, and TxA2 and PGE2 (20, 22, 17). These two prostaglandins function as local hormones or autacoids acting on G protein-coupled receptors that mediate the sustained or tonic contraction.

GRANTS

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