Reactive oxygen species (H₂O₂): effects on the gallbladder muscle of guinea pigs

ZUO-LIANG XIAO,1 MARIA J. POZO ANDRADA,2 PIERO BIANCANI,1 AND JOSE BEHAR1
1Department of Medicine, Rhode Island Hospital and Brown University School of Medicine, Providence, Rhode Island 02903; and 2Avda Universidad s/n Nursing School, 10071 Caceres, Spain

Received 6 June 2001; accepted in final form 1 November 2001

Xiao, Zuo-Liang, Maria J. Pozo Andrades, Piero Biancani, and Jose Behar. Reactive oxygen species (H₂O₂): effects on the gallbladder muscle of guinea pigs. Am J Physiol Gastrointest Liver Physiol 282: G300–G306, 2002. First published October 24, 2001; 10.1152/ajpgi.00241.2001.—Reactive oxygen species (ROS) have been implicated in the pathogenesis of muscle dysfunction in acute inflammatory processes. The aim of these studies was to determine the effects of ROS on gallbladder muscle function in vitro. Single muscle cells were obtained by enzymatic digestion. H₂O₂ (70 μM) caused maximal contraction of up to 14% and blocked the response to CCK-8, ACh, and KCl. It did not affect the contractions induced by guanosine 5′-O-(3-thiotriphosphate), diacylglycerol, and inositol 1,4,5-trisphosphate that circumvent membrane receptors. The contraction induced by H₂O₂ was inhibited by AACOCF₃ [cytosolic phospholipase A₂ (cPLA₂) inhibitor], indomethacin (cyclooxygenase inhibitor), chelerythrine [protein kinase C (PKC) inhibitor], or PD-98059 [mitogen-activated protein kinase (MAPK) inhibitor]. H₂O₂ also reduced the PKC receptor binding capacity from 0.36 ± 0.05 pmol/mg protein (controls) to 0.17 ± 0.03 pmol/mg protein. The level of lipid peroxidation as well as the PGE₂ content was significantly increased after H₂O₂ pre-treatment. Unlike superoxide dismutase, the free radical scavenger catalase prevented the H₂O₂ induced contraction, and its inhibition of the CCK-8 induced contraction. It is concluded that ROS cause damage to the plasma membrane of the gallbladder muscle and contraction through the generation of PGE₂ induced by cPLA₂-cyclooxygenase and probably mediated by the PKC-MAPK pathway.

MATERIALS AND METHODS

Animals. Adult male guinea pigs were purchased from Elm Hill Breeding Laboratory (Chelmsford, MA). The Animal Welfare Committee of Rhode Island Hospital approved their use. Animals were housed in thermoregulated rooms and had free access to food and water. After an overnight fast, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg) followed by pentobarbital sodium (30 mg/kg ip). The gallbladder was removed and rinsed with ice-cold, oxygenated Krebs solution (in mM: 116.6 NaCl, 3.4 KCl, 21.9 NaHCO₃, 1.2 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, and 5.4 glucose). The mucosa and muscle strips from various segments of guinea pig stomach exhibited different responses to PGE₁, PGE₂, and PGF₂α (22). In rat stomach, PGE₂ caused a contraction in the longitudinal preparation but had no effect on the circular muscle (35). PGE₁, PGE₂, and PGF₂α potentiated the contractile responses of guinea pig gallbladder to transmural stimulation and ACh (28). Prostaglandins may also be involved in muscle cytoprotection since PGE₂ and other prostaglandins appear to participate in the pathway responsible for increased formation of scavengers of free radicals (2). These studies therefore were aimed at examining the effects of ROS on gallbladder muscle function utilizing H₂O₂ on enzymatic digested single muscle cells from normal guinea pig gallbladders.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
serosa were carefully peeled off under a dissecting microscope. The muscle layer was further cleaned by gently removing the remaining connective tissue.

**Isolation and permeabilization of muscle cells.** Single muscle cells were obtained by enzymatic digestion (42, 45–47). Gallbladder 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 U/mg protein) for 20 min at 35°C in a shaking water bath. The buffer was gently gassed with 100% O₂ during digestion. At the end of the digestive process, the tissue was filtered through a Nitex mesh 200 (Tetko, Elmsford, NY) and rinsed with 20 ml HEPES. The tissue remaining on the filter was collected and incubated in HEPES buffer at 35°C for 15 min to allow the free dispersion of cells.

Muscle cells were permeabilized with saponin. The partially digested muscle layer was rinsed with “cytotoxic buffer” (4, 42, 45–47). After cells were dispersed, the cell suspension was briefly treated with saponin (75 μg/ml) during centrifugation at 200 g for 3 min. Cells were washed and resuspended in modified cytosolic buffer for further use.

**Studies on muscle cell contraction.** Muscle contraction was determined in cells treated with increasing concentrations of H₂O₂ for variable periods of time before treatment with CCK-8 (10 nM), ACh (10 μM), guanosine 5’-O-(3-thiotriphosphate) (GTPγS; 10⁻⁵ M), diacylglycerol (DAG; 10⁻⁷ M), and inositol 1,4,5-trisphosphate (IP₃; 10⁻⁶ M). Cells were measured in cell suspensions as described previously. The cells were allowed to react with the agonists for 30 s and then fixed by adding acrolein (4, 42, 45–47). Contraction was expressed as the mean of the percent shortening of 30 individual cells with respect to control (i.e., untreated) cells.

**Preparation of plasma membranes.** Plasma membranes were prepared and purified by sucrose gradient centrifugation as described before (34, 40). H₂O₂-treated and untreated muscle cells were homogenized separately by using a tissue-tearer (Biospec Products, Racine, WI) in 10 volumes by weight of a sucrose-HEPES buffer. The homogenates were centrifuged at 600 g for 5 min, and the supernatant was collected in a clean centrifuge tube (Beckman Instruments) and centrifuged at 150,000 g for 45 min. The pellet was resuspended in sucrose-HEPES, layered over a linear 9–60% sucrose gradient, and centrifuged at 90,000 g for 3 h. The plasma membranes were collected at ~24% sucrose. They were then diluted and pelleted by centrifugation at 150,000 g for 30 min. The pellet of membranes was stored at ~70°C.

**225I-labeled CCK-8 binding to plasma membranes.** Ligand-binding experiments were performed in a final volume of 300 μl (33, 36, 40). Membranes containing 50 μg of protein were incubated with 50 pM of 225I-labeled CCK-8 for 30 min to favor binding. The suspension was diluted and added to polystyrene counting vials. Radioactivity remaining on the filters was counted. The results are expressed as specific binding (% total activity added to incubation volume) achieved by subtracting nonspecific binding (in the presence of 10⁻⁷ M unlabeled CCK) from total binding to membranes.

**Scatchard analysis.** A computer analysis of the ligand-fitting program (26) based on the displacement curve was used to obtain the maximal binding capacity of CCK receptors.

**Assessment of lipid peroxidation.** Purified plasma membranes were resuspended with 1.15% KCl (30) and mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution (pH 3.5), and 1.5 ml of 0.8% aqueous solution of 2-thiobarbituric acid. This mixture was added to a volume of 4 ml with distilled water. The sample was heated at 95°C for 60 min. After cooling, 1 ml of distilled water and 5 ml of the mixture of n-butanol and pyridine (15:1 vol/vol) were added and shaken vigorously. The organic layer was taken after it was spun at 2,000 g for 10 min, and its absorbance (red pigment) was measured at 532 nm. 1,1,3,3-tetramethoxy-propane was used as an external standard. The level of lipid peroxides was expressed as nanomoles of malonaldehyde (MDA) per 100 mg protein. (MDA is a secondary product of lipid peroxidation.)

**Measurements of PGE₂ content.** The PGE₂ content was measured by using a radioimmunoassay kit from NEN (Boston, MA) (9). H₂O₂-treated and untreated muscle cells were homogenized in HEPES buffer containing EDTA/γ-dimethylacine to inhibit the metabolism of arachidonic acid to prosta- glandins. The suspension was centrifuged at 10,000 g for 15 min. PGE₂ was extracted from the supernatant by the method of Kelly et al. (19). Extracted PGE₂ was converted into its methyl oximate derivative using the methyl oximation reagent. The PGE₂ content was determined by following the kit’s protocol and expressed as picograms per milligram protein.

**Protein determination.** The protein content of the muscle membranes was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY). Values for each sample were means of triplicate measurements.

**Drugs and chemicals.** H₂O₂ was obtained from Fisher. 225I-labeled CCK-8 and PGE₂ radioimmunoassay kits were obtained from NEN. Type F collagenase, papain, and other reagents were purchased from Sigma (St. Louis, MO).

**Data analysis.** One- and two-factorial repeated ANOVA and unpaired Student’s t-test were used for statistical analysis. P < 0.05 was considered statistically significant.

**RESULTS**

Single muscle cells of guinea pig gallbladders were obtained by enzymatic digestion. The average resting lengths of intact and permeabilized cells were 59.8 ± 1.5 and 59.1 ± 1.2 μm (n = 4), respectively. The resting length of control cells treated with buffer was 59.6 ± 0.7 μm. No significant differences in resting cell length were observed among them (one-factor ANOVA).

H₂O₂ induced muscle contraction in a time-dependent manner (Fig. 1). The contraction developed shortly after the cells were incubated with 70 μM H₂O₂, peaked at 15 min (14.3 ± 0.4% shortening), and lasted for at least 60 min. H₂O₂ at a concentration of 70 μM also time-dependently blocked CCK-8-induced contraction (Fig. 1). In these experiments, muscle cells were incubated with either buffer or H₂O₂ (70 μM) for increasing periods of time (5, 10, 15, 30, 45, and 60 min). Then aliquots of buffer or H₂O₂-treated cells were exposed to buffer or CCK-8 (10 nM) for 30 s. There were no significant differences in CCK-induced contraction in muscle cells pretreated with buffer for increasing periods of time ranging between 19 and 22% (Fig. 1).

In contrast, after exposure to H₂O₂, the CCK-8-induced contraction was gradually and significantly reduced from 21.3 ± 0.6% (CCK-8 alone, control) to 14.9 ± 0.9% at 15 min (*P < 0.001 by Student’s t-test). The magnitude of this response to CCK was not different from that of H₂O₂ alone (Fig. 1).
The H2O2-induced muscle contraction was also concentration dependent (Fig. 2A). After incubation for 15 min, the H2O2-induced contraction increased from 4.6 ± 0.2% after 17.5 μM to a peak of 14.2 ± 1.1% after 70 μM. In contrast, the CCK-8-induced contraction was gradually reduced by increasing the concentrations of H2O2 to a level similar to the H2O2-induced contraction. The contraction induced by H2O2 was completely blocked by pretreating the muscle cells with the cyclooxygenase inhibitor indomethacin for 15 min at a dose of 5 μM (*P < 0.001 by ANOVA). These data indicate that increased prostaglandin synthesis may be involved in H2O2-induced muscle contraction. In this experiment, muscle cells were exposed to CCK-8 for only 30 s (*P < 0.001 vs. CCK-8 alone by Student’s t-test). Values are means ± SE of 3 experiments.

The H2O2-induced muscle contraction was also concentration dependent (Fig. 2A). After incubation for 15 min, the H2O2-induced contraction increased from 4.6 ± 0.2% after 17.5 μM to a peak of 14.2 ± 1.1% after 70 μM. In contrast, the CCK-8-induced contraction was gradually reduced by increasing the concentrations of H2O2 to a level similar to the H2O2-induced contraction. The contraction induced by H2O2 was completely blocked by pretreating the muscle cells with the cyclooxygenase inhibitor indomethacin for 15 min at a dose of 5 μM (*P < 0.001 by ANOVA). These data indicate that increased prostaglandin synthesis may be involved in H2O2-induced contraction. Because H2O2 causes muscle contraction by itself, the full inhibitory effect of this free radical on the CCK-8-induced contraction was determined by pretreating muscle cells with both indomethacin and H2O2. Under these conditions, the action of CCK-8 was completely blocked, suggesting that the free radical inhibits the contraction of this peptide. Moreover, the dose-response relationship of CCK was unaffected by pretreatment with indomethacin (Fig. 2B).

In control conditions, the receptor-dependent agonists ACh and PGE2 and the receptor-independent agonist KCl maximally contracted muscle cells by ~20% (Fig. 3). Pretreatment with H2O2 decreased ACh- and KCl-induced contraction to 14.2 ± 1.3 and 13.2 ± 0.8%, respectively (*P < 0.01 and **P < 0.001 by Student’s t-test), which were not statistically different from the contraction induced by H2O2 alone. However, PGE2-induced contraction was unaffected by H2O2.

To examine the cellular site of H2O2 inhibition of the agonist-induced muscle contraction, the G protein activator GTPγS and the second messengers DAG and IP3 were used (Fig. 4). Pretreatment of muscle cells with H2O2 had no effect on GTPγS, DAG-, or IP3-induced contraction. These data suggest that H2O2-induced muscle damage is located in the plasma membrane.

Binding studies were performed to localize more precisely the site of H2O2 action (Fig. 5). The maximal specific binding of 125I-labeled CCK-8 to CCKA receptors in plasma membranes from control gallbladder muscle was 11.8 ± 0.3%, which was reduced to 5.8 ± 0.7% by pretreatment with 70 μM H2O2 for 15 min. Scatchard analysis showed that the binding capacities of CCK receptors in plasma membrane from normal and H2O2-pretreated gallbladder muscle were 0.36 ±
0.05 and 0.17 ± 0.03 pmol/mg protein, respectively (P < 0.001 by Student’s t-test).

Measurements of lipid peroxidation were performed because membrane phospholipids are susceptible to damage by free radicals (Fig. 6). The level of lipid peroxidation in buffer-treated cells was 182 ± 48 nmoL MDA/100 mg protein, which increased to 332 ± 42 nmoL MDA/100 mg protein after H2O2 treatment (*P < 0.01 by Student’s t-test). These data suggest that membrane phospholipids are an additional site of H2O2 injury.

As mentioned above, muscle cells contract when acutely exposed to H2O2. The mechanisms responsible for this contraction are not known. To investigate the pathways involved in this contraction, a series of antagonists were used. H2O2-induced muscle contraction was blocked by inhibitors of phospholipase A2 (cPLA2), cyclooxygenase, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (Fig. 7). AACOCF3 (cPLA2 inhibitor) completely blocked the contraction caused by H2O2 from control values of 14.2 ± 3.2 to 0.9 ± 0.6%; chelerythrine (PKC inhibitor) and indo-methacin (cyclooxygenase inhibitor) decreased H2O2-induced contraction from control values of 14.2 ± 3.2 to 2.5 ± 1.3 and 3.5 ± 1.4%, respectively. PD-98059 (MAPK inhibitor) blocked H2O2-induced contraction from control values of 14.2 ± 3.2 to 0.8 ± 0.6% (*P < 0.001 by Student’s t-test). These data indicate that the cPLA2, cyclooxygenase, PKC, and MAPK pathways are involved in the contraction elicited by H2O2.

This hypothesis is supported by the finding that there is an increase in the PGE2 content in muscle cells treated with H2O2 to 14.5 ± 2.7 from 7.1 ± 1.6 ng/mg protein in buffer-treated controls (*P < 0.01) (Fig. 8). This increase in PGE2 content caused by H2O2 was prevented by pretreatment with the free radical scavenger catalase but was unaffected by superoxide dismutase (SOD; Fig. 8).
We then examined whether scavengers of free radicals prevent the muscle contraction and damage caused by H$_2$O$_2$ (Fig. 9). SOD had no effect on H$_2$O$_2$-induced contraction or on its inhibition of CCK-8-induced contraction. In contrast, catalase eliminated the H$_2$O$_2$-induced contraction ($^{* *}$P < 0.001 by Student’s t-test) and the inhibition of H$_2$O$_2$ on CCK-8-induced contraction ($^{* * *}$P < 0.001 by Student’s t-test).

These studies therefore demonstrate some of the steps in the pathway that mediates the H$_2$O$_2$-induced muscle contraction (Fig. 10). H$_2$O$_2$ appears to initially act on plasma membranes to cause lipid peroxidation and generate hydroperoxides that activate cPLA$_2$ to stimulate arachidonic acid hydrolysis and release PGE$_2$ through cyclooxygenase. PGE$_2$ may translocate to the membrane and bind to receptors to cause muscle contraction by utilizing the PKC and MAPK pathways.

**DISCUSSION**

Activated polymorphonuclear cells are the main source of ROS (6, 39) that damage plasma membranes of epithelial and smooth muscle cells (1, 3, 7). It has been shown that polymorphonuclear cells release NH$_2$Cl, which is converted by SOD to H$_2$O$_2$ and then inactivated by catalase (20, 21). H$_2$O$_2$ can act directly on plasma membranes or readily cross the membranes and give rise to more radical species inside the cells, where they affect several sites (20). ROS have been shown to disrupt intestinal motility, causing an initial contraction followed by a slow relaxation as well as blocking the contraction induced by methacholine (37). They also inhibit the gallbladder muscle contraction.
induced by CCK-8, ACh, and KCl in muscle strips (10, 23, 25).

The mechanisms whereby ROS cause muscle contraction and inhibit agonist-induced contraction have not been elucidated. Our studies showed that, when normal muscle cells are briefly exposed to H2O2 in vitro, it results in: 1) muscle contraction of up to 14%, 2) inhibition of membrane-dependent agonist-induced contraction, probably by oxidizing the sulfhydryl groups of transmembrane receptors, transport proteins, and calcium channels (8, 20), 3) a decrease in the binding capacity of CCK receptors, and 4) a normal response to agonists such as DAG, IP3, and GTPγS that bypass the membrane receptors and calcium channels. These findings suggest that the initial injury is localized in the plasma membrane, with the contractile apparatus remaining functionally intact.

The damage to the plasma membrane is also supported by the finding that there is an increase in the level of lipid peroxidation, as demonstrated by the greater production of TBA metabolites. This finding may explain the depletion of membrane phospholipids that could result in changes in membrane fluidity and impair further the functions of membrane proteins (1, 11, 38). ROS also stimulate a cytoprotective response that includes an increase in the production of PGE2, which in turn generates scavengers of free radicals such as SOD and catalase. Furthermore, by increasing the synthesis of PGE2, H2O2 causes muscle contraction through the activation of PKC and MAPK (15, 18, 24, 27, 31). The mechanisms whereby ROS stimulate the cPLA2–PGE2 pathway are unknown. Our preliminary studies suggest that oxidized phospholipid byproducts such as platelet-activating factor-like lipids may mediate this response (14). Moreover, in agreement with previous reports, these H2O2 effects are prevented by the free radical scavenger catalase but were unaffected by SOD in vitro (15, 44).

The findings that ROS increase the PGE2 content and that indomethacin blocks the H2O2-induced contraction suggest that prostaglandins mediate the contraction induced by free radicals. We cannot exclude the involvement of other prostaglandins such as PGF2α and thromboxane A in the muscle response to H2O2. These studies, however, were focused on the relationship between PGE2 and H2O2, mainly because this prostaglandin may play an important role in protecting muscle cells against oxidative stress (43).

Acute inflammatory processes may contribute to the pathogenesis of muscle dysfunction by increasing the generation of ROS. The finding that ROS reproduce, in normal muscle cells, most of the abnormalities found in muscle cells from specimens affected by acute cholecystitis supports this conclusion (41). These abnormalities are characterized by 1) inhibition of the contraction induced by agonists that are membrane dependent (CCK, ACh, KCl) without affecting the actions of GTPγS, DAG, and IP3 that circumvent membrane receptors; 2) depletion of membrane phospholipids, increased cholesterol-to-phospholipid ratio, and lipid peroxidation, which could decrease the membrane fluidity; 3) lower

125I-labeled CCK-8 binding capacity; and 4) increase in PGE2 levels and SOD and catalase activities. Therefore, the muscle abnormalities observed in acute cholecystitis may be due to the actions of ROS on muscle cells (23). The preservation of the PGE2-induced contraction suggests that the increased production of this eicosanoid may protect its membrane receptors (13). This protective mechanism may be involved in muscle cytoprotection because PGE2 and other prostaglandins appear to stimulate the generation of scavengers of free radicals (2). However, further studies are needed to determine the mechanisms and functions of this receptor protection.

In summary, H2O2 appears to affect constituents of the plasma membrane of muscle cells. It impairs the contraction caused by agonists by damaging their receptors. It also affects membrane phospholipids, causing lipid peroxidation, which activates cPLA2 and cyclooxygenase to produce PGE2. PGE2 binds to its receptors, causing muscle contraction. The mechanism whereby the PGE2-induced contraction was protected from H2O2 needs to be further investigated.

These data were partially presented at the Annual Meeting of the American Gastroenterological Association, Orlando FL, May 1999. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-27389.

REFERENCES


