Role of PGE$_2$ on gallbladder muscle cytoprotection of guinea pigs

Zuo-Liang Xiao, Piero Biancani, and Jose Behar

Department of Medicine, Rhode Island Hospital and Brown University School of Medicine, Providence, Rhode Island 02903

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**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.

**Isolation of muscle cells.** Single muscle cells were obtained by enzymatic digestion (41, 48–50). The GB muscle layer was cut into 2-mm-wide strip 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 gassed gently with 100% O$_2$ 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 (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.

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ROS contract GB muscle cells by themselves but impair their response to CCK-8, ACh, and KCl without affecting the actions of PGE$_2$. The muscle contraction induced by ROS and TCDC is blocked by indomethacin or by a nonspecific membrane receptor inactivator (N-ethylmaleimide), suggesting that their actions are mediated by prostaglandins acting on membrane receptors (44, 45). H$_2$O$_2$ and TCDC also increase the generation of SOD and catalase by activating the PGE$_2$-PKC-MAPK pathway (44, 45). However, the fact that the functions of PGE$_2$ are impaired in GB with lithogenic bile with excessive cholesterol (Ch; see Ref. 38) suggests that excessive Ch affects the functional integrity of its receptors and therefore the cytoprotective response to ROS.

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KCl (20 mM). In another group, cells were pretreated with buffer (controls) or PGE2 before incubation with H2O2 or TCDD followed by agonist stimulation. In some studies, muscle cells were pretreated with Ch-rich liposomes for 4 h, which allows excessive Ch to incorporate in the plasma membrane (47). The cell length was measured in cell suspensions, as described previously. They were allowed to react with the agonists for 30 s and then fixed by adding acrolein (5, 41, 48–50). Contraction was expressed as the mean of the percentage of 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 previously (31, 40). Muscle cells were homogenized by using a tissue tearer (Biospec Products, Racine, WI) in 10 vol 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.

Studies of receptor desensitization. Muscle cells were treated with either CCK-8 or PGE2 for 60 min followed by binding experiments performed after all traces of CCK-8 or PGE2 were removed by repeated washing (30, 32, 40, 42). Cells were then incubated with 125I-labeled CCK-8 or [3H]PGE2 at 25°C. At different time periods, aliquots of cells were collected, and the separation of bound from free radioligand was achieved by filtration using a vacuum-filtering manifold (Millipore). Radioactivity remaining on the filters was counted. The results are expressed as specific binding (%total activity added to the incubation volume) achieved by subtracting nonspecific binding (in the presence of 10−7 M unlabeled CCK or 10−6 M unlabeled PGE2) from total binding to membranes.

Assessment of lipid peroxidation. Purified plasma membranes were resuspended with 1.15% KCl (28) 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 of distilled water. The sample was heated at 95°C for 60 min. After cooling, 1 ml 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-Tetramethoxypropane was used as an external standard. The level of lipid peroxides was expressed as nanomoles of malondialdehyde per milligram protein.

Measurement of catalase activity. The catalase activity was determined by using the method reported by others (8, 26). Muscle cells were homogenized in HEPES buffer, and the suspension was centrifuged at 10,000 g for 15 min. Duplicate 25-μl aliquots of each sample of the supernatant and 75 μl of 10 mM phosphate buffer, pH 7.4, were placed in 3-ml cuvettes. Duplicate blanks (B) of a solution containing 90 μl phosphate buffer, 10 μl of 1% sodium azide, 200 μl of 6 N sulfuric acid, and 1 ml of 6 mM H2O2 were also prepared. The enzymatic reactions were initiated sequentially by fixing intervals by adding 1 ml of 6 mM H2O2 to the samples. After 3 min, the reactions were stopped sequentially at the same fixed intervals by rapidly adding and mixing 200 μl of 6 N H2SO4. Finally, 1.4 ml of 0.01 N KMnO4 was added to each cuvette and mixed thoroughly. A spectrophotometric standard (S0) was prepared by adding 7.0 ml of 0.01 N KMnO4 to a mixture of 5.5 ml phosphate buffer and 1.0 ml of 6 N H2SO4. The absorbency of the solution was read at 480 nm within 30–60 s after the addition of KMnO4. The unit of catalase activity (k) was defined as the amount of enzyme that consumed 1 μmol H2O2·mg protein−1·min−1. Calculation of the catalase activity was achieved according to the equation: k = (log(S0/Sf)) × 2.33/t, where S0 was obtained by subtracting the absorbency of B from the Sf; Sf was obtained by subtracting the absorbency of the samples from S0; and t = 3 min in this experiment. Data were expressed as units per milligram protein.

Measurement of SOD activity. The total SOD activity was measured by using a spectrophotometric assay kit (R&D Systems, Minneapolis, MN; see Ref. 25). Muscle cells were homogenized in HEPES buffer. The supernatant was obtained after the suspension was centrifuged at 8,500 g for 10 min at 4°C. Ice-cold extraction reagent (400 μl; 62.5:37.5 ethanol-chloroform, vol/vol) was added to 250 μl of the supernatant, vortexed for at least 30 s, and centrifuged at 3,000 g for 10 min at 4°C. Next, the aqueous upper layer was collected for assay of the SOD activity. The determination of SOD activity was achieved by following the kit’s protocol. Data were expressed as units 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; 125I-CCK-8, [3H]PGE2, and the PGE2 RIA kit were obtained from NEN Life Science Products (Boston, MA). TCDC, type F collagenase, papain, and other reagents were purchased from Sigma Chemical (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 to be statistically significant.

RESULTS

Enzymatically dissociated muscle cells were treated with buffer or H2O2 for 15 min followed by stimulation with an agonist for 30 s. Buffer-treated muscle cells contract or shorten an average of 20% in response to agonists (42). The percent decrease in cell length of buffer-treated groups in response to CCK-8, ACh, and KCl was 20.3 ± 1.0, 21.3 ± 0.6, and 20.5 ± 1.3%, respectively. Pretreatment with H2O2 (70 μM) inhibited the contraction induced by CCK-8, ACh, and KCl (Fig. 1) and was not significantly different from the contraction induced by H2O2 alone. H2O2 alone caused muscle cell contraction up to 14.2 ± 1.1% (44). However, PGE2-induced contraction was not affected by H2O2. Muscle cells pretreated with PGE2 for 15 min before exposure to H2O2 prevented the inhibitory actions of H2O2 on CCK-8, ACh-, and KCl-induced contraction (P < 0.01 vs. H2O2-treated group).

Fig. 1. Effect of PGE2 pretreatment on H2O2 inhibition of muscle contraction in response to agonists. Incubation with H2O2 (70 μM) for 15 min inhibited the contraction induced by CCK-8, ACh, and KCl (*P < 0.01) but did not antagonize the contraction caused by PGE2. Pretreatment with PGE2 for 15 min followed by H2O2 prevented the inhibitory actions of H2O2 on agonist-induced contraction.
Similar results were obtained in muscle cells treated with buffer or TCDC for 15 min followed by stimulation with agonists for 30 s (Fig. 2). The percent decrease in cell length of buffer-treated groups in response to CCK-8, ACh, and KCl was 20.3 ± 1.0, 21.3 ± 0.6, and 20.5 ± 1.3%, respectively. Pretreatment with TCDC inhibited the contraction induced by CCK-8, ACh, and KCl without affecting the action of PGE2. Preincubation of muscle cells with PGE2 for 15 min before their exposure to TCDC prevented its inhibitory actions on these agonists (*P < 0.05 vs. TCDC-treated group). These data suggest that PGE2 participates in the cytoprotective mechanisms used by muscle cells against H2O2 or TCDC.

The level of lipid peroxidation in plasma membranes of muscle cells increases after exposure to H2O2 and TCDC (44). The levels of lipid peroxidation were measured after the cells were pretreated with either buffer (control) or PGE2 for 15 min before the addition of H2O2 and TCDC (Fig. 3) to determine whether PGE2 could prevent the increase in lipid peroxidation. The basal level of lipid peroxidation in control was 245 ± 18 nmol MDA/100 mg protein and was not affected by pretreatment with PGE2 for 3 h. PGE2 pretreatment for 3 h before TCDC for 30 min, however, decreased the expected increase in lipid peroxidation caused by TCDC (P < 0.01). Pretreatment with PGE2 for 3 h followed by H2O2 for 30 min also reduced the expected increase in the levels of lipid peroxidation caused by H2O2 (P < 0.001). These data suggest that PGE2 pretreatment is capable of protecting muscle cells from the damage caused by H2O2 and TCDC.

Our previous studies have shown that TCDC increases the generation of H2O2 (45) and that catalase inactivates free radicals by converting H2O2 to H2O. Thus it is conceivable that the cytoprotective mechanisms of PGE2 may be mediated by catalase (10). Therefore, the catalase activity was measured in muscle cells treated with PGE2 for 3 h before H2O2 and TCDC (Fig. 4). PGE2 alone increased the catalase activity (P < 0.001). H2O2 and TCDC by themselves also increased the levels of catalase activity and were prevented by pretreatment with PGE2 before H2O2 and TCDC (P < 0.001 and P < 0.01, respectively). The reason for the expected increase in the levels of catalase activity after PGE2 pretreatment before H2O2 and TCDC may be because of the consumption of catalase by the added H2O2 or generation of H2O2 caused by TCDC. Inactivated free radicals also do not stimulate catalase activity.

The level of SOD activity was also stimulated by PGE2 treatment for 3 h (Fig. 5). SOD activity in the resting state was 2.7 ± 0.1 U/mg protein. PGE2 increased the SOD activity (P < 0.001). H2O2 and TCDC also increased the level of SOD activity, which was reduced by pretreatment with PGE2 before H2O2 and TCDC (P < 0.05 and P < 0.05, respectively).

Agonist stimulation may desensitize its own receptors for a variable period of time and affect the receptor function to a second agonist stimulation (11). Our previous data have shown that the production of PGE2 in inflammatory conditions is continuous and the increased catalase activity induced by PGE2 is not affected in a prolonged period (42, 44). These data suggest that the function of PGE2 receptors is intact in the presence of PGE2. We therefore examine whether the cytoprotective role of PGE2 was affected by receptor desensitization by comparing its desensitization time with that of CCK-8. Muscle cells were treated with buffer alone (controls) or with CCK-8 for 60 min followed by CCK receptor binding studies determined at different time periods after the cells were washed off of all traces of CCK-8 (Fig. 6). Sixty minutes of incubation time are necessary for the binding of CCK receptors to reach a dynamic balance. Muscle cells were then incubated with 125I-

Fig. 2. Effect of PGE2 pretreatment on taurocholodeoxycholic acid (TCDC) inhibition of muscle contraction in response to agonists. TCDC (50 μM) inhibited the contraction induced by CCK-8, ACh, and KCl (*P < 0.05) without affecting the contraction caused by PGE2. Pretreatment with PGE2 for 15 min followed by TCDC prevented the inhibitory action of TCDC on these agonists.

Fig. 3. Effect of PGE2 pretreatment on lipid peroxidation [expressed as malonaldehyde (MDA)] caused by TCDC or H2O2. Muscle cells were pretreated with PGE2 for 3 h followed by treatment with TCDC or H2O2 for 30 min. TCDC or H2O2 alone increased the magnitude of lipid peroxidation, which was prevented by pretreatment with PGE2 (*P < 0.01 and **P < 0.001).

Fig. 4. Effect of PGE2, H2O2, and TCDC on catalase activity in muscle cells. The increase in catalase activity induced by H2O2 and TCDC was prevented by pretreatment for 3 h with PGE2 (*P < 0.001 and **P < 0.01).
CCK-8 for different time intervals. At 2 min, the total binding of \(^{125}\text{I}-\text{CCK-8}\) to its receptors was not different between the control and CCK-8-treated groups. After 4 min, the total binding in the CCK-8-treated group is 1.2 ± 0.2% and was significantly lower than 4.1 ± 0.3% in the control group (\(P < 0.05\)). At 15 min, the total binding in the CCK-8-treated group had almost recovered but still was lower than that in the control group (\(P < 0.05\)). The magnitude of \(^{125}\text{I}-\text{CCK-8}\) bound to its receptors in the CCK-8-treated cells returned to normal levels (which were not different from the control group) after 20–30 min of incubation time.

In muscle cells pretreated with PGE\(_2\) for 60 min (Fig. 7), however, the binding of [\(^3\text{H}\)]PGE\(_2\) to its receptors was not significantly different between the two groups at any time point. At 2 min, both PGE\(_2\)-treated and buffer-treated muscle cells reached a total binding of 2.6 ± 0.2 and 2.4 ± 0.3%, respectively. After 10 min, the binding of PGE\(_2\) to its receptors reached a plateau of 9.0 ± 0.6 and 8.5 ± 0.3%. These data suggest that PGE\(_2\) receptors have no significant desensitization period in the presence of its agonist.

Previous studies have shown that muscle contraction induced by CCK-8, ACh, KCl, and PGE\(_2\) is impaired in human GB with lithogenic bile with Ch (6). This defective contraction is the result of a dysfunction of membrane receptors and ion channels caused by the excessive Ch incorporation by plasma membranes (40). We therefore examined whether PGE\(_2\)-mediated cytoprotection against free radicals was impaired, since PGE\(_2\) receptors mediate the generation of scavengers of free radicals. Normal muscle cells were incubated with either buffer or Ch-rich liposomes for 4 h. They were then treated with PGE\(_2\) for 3 h before the addition of H\(_2\)O\(_2\) (Fig. 8). The Ch content in the plasma membranes is increased after incubation with Ch-rich liposomes (47). Excessive Ch in the plasma membranes reduced agonist-induced contraction of CCK-8, ACh, KCl, and PGE\(_2\) (\(P < 0.001\) vs. respective Ch-rich liposomes treated alone). The addition of H\(_2\)O\(_2\) to these cells further decreased CCK-8-, ACh-, and KCl-induced contraction (\(P < 0.01\)) without further decreasing PGE\(_2\)-induced contraction. Pretreatment with PGE\(_2\) before H\(_2\)O\(_2\) in Ch-rich lipo-
some-treated cells only partially prevented the inhibition of 
H2O2 on agonist (CCK-8, ACh, and KCl)-induced contraction 
(P < 0.05 vs. Ch-rich liposomes treated alone). These data 
suggest that PGE2 receptors are affected by the excessive Ch 
incorporation in the plasma membrane and therefore are unable 
to provide full cytoprotection to muscle cells from the actions 
of H2O2.

Similar results were obtained in normal muscle cells incu-
bated with Ch-rich liposomes followed by TCDC treatment 
(Fig. 9). Compared with those treated with buffer, incubation 
with Ch-rich liposomes reduced agonist-induced contraction 
of CCK-8, ACh, KCl, and PGE2 (P < 0.01). The addition of 
TCDC further reduced the CCK-8-, ACh-, and KCl-induced 
contraction without further affecting the contraction induced 
by PGE2. Pretreatment with PGE2 for 3 h before TCDC did not 
fully prevent the inhibition of TCDC on agonist (CCK-8, ACh, 
and KCl)-induced contraction (P < 0.05).

Catalase activity stimulated by PGE2, H2O2, and TCDC was 
also markedly reduced in muscle cells pretreated with Ch-rich 
liposomes compared with Ch-free liposomes or buffer (Fig. 10, 
P < 0.05 to 0.001).

DISCUSSION

Our results showed that pretreatment with PGE2 prevented 
the expected muscle abnormalities induced by H2O2 and 
TCDC. PGE2 prevented the inhibitory effects of H2O2 and 
TCDC on agonist-induced muscle contraction and the expected 
increase in lipid peroxidation and activities of free radical 
scavengers induced by H2O2 and TCDC (44, 45). PGE2 seems 
to be able to carry out these cytoprotective actions by stimu-
lating rapidly (15 min) and prolonged (3 h) the activities of 
SOD and catalase. These studies also showed that there is no 
significant desensitization for PGE2 receptors compared with 
the desensitization period for CCK receptors. This period of 
desensitization for CCK is in agreement with the previous 
studies showing that the recycling time for CCK-1 receptors is 
between 20 and 60 min (34). The very short desensitization 
period of PGE2 receptors may contribute to the muscle cyto-
protection by continuously stimulating the generation of scav-
engers of free radicals. These differences between CCK-1 and 
PGE2 receptors may be the result of a different type or process 
of receptor internalization (15, 46) or even no receptor inter-
nalization (20, 35) because they may have a rapid recycling 
half-time of as low as 1.5 min (13). It is conceivable that PGE2 
receptors may be included in the latter type of receptors.

However, the actions of PGE2 are impaired by higher Ch 
content in the plasma membrane that affects all membrane 
receptors and ion channels studied, including PGE2 receptors 
(7, 38). This observation explains the reduced muscle contrac-
tion and catalase activities induced by H2O2 and TCDC in 
muscle cells incubated with Ch-rich liposomes, since they are 
mediated by PGE2 receptors. These findings may also explain 
the inability of PGE2 to fully reverse the effects of oxidative 
stress on Ch-rich liposome-treated cells.

Activated polymorphonuclear cells are the main source of 
ROS (9, 37) that damage plasma membranes of epithelial and 
smooth muscle cells (3, 4, 9). Polymorphonuclear cells release 
NH2Cl that is converted by SOD to H2O2 and then are 
inactivated by catalase (17, 18). Muscle cells can also generate 
ROS when subjected to injury. TCDC damages protein consti-
tuents of plasma membranes by stimulating the generation of 
H2O2 (45). The damage caused by oxidative stress is probably 
attenuated and limited to the plasma membrane by the 
increased production of PGE2 and of scavengers of free radicals, 
such as SOD and catalase (44).

By increasing the synthesis of PGE2, H2O2 causes muscle 
contraction and increases the activities of scavengers of free 
radicals through the PKC-MAPK pathway (12, 16, 21, 22, 29). 
PGE2 plays a role in the contraction induced by H2O2 and 
TCDC, since it is blocked by the cyclooxygenase inhibitor 
indomethacin, suggesting that the actions of H2O2 and TCDC 
are mediated in part by cyclooxygenase byproducts that are 
also important participants in the inflammatory process (14, 
44). These observations are in agreement with previous find-
ings in that the tracheal and ileal muscle contraction induced by 
H2O2 in vitro appears to be mediated by prostaglandins 
(21, 27).

The GB appears to protect itself from the deleterious actions 
of hydrophobic bile acids and other constituents (1) by periodic 
emptying of bile during the interdigestive and postprandial 
periods and by cytoprotective mechanisms of epithelial and 
muscle cells. GB emptying in the interdigestive period occurs
during phase II of the migrating motor complex and during the digestive period stimulated by CCK. Both stimuli are mediated by cholinergic mechanisms (1). The inhibition of the CCK- and ACh-induced contraction caused by TCDC and ROS in vivo worsens the stasis and further increases the deleterious effects of bile constituents (44). GB stasis may therefore be one of the factors that facilitates the development of acute cholecystitis (33).

The contraction of normal muscle cells induced by PGE2 is preserved after they are exposed to H2O2 and TCDC, even though these stimuli stimulate the activities of scavengers of free radicals (4, 11). Signal transduction pathways may play a cytoprotective role (4). This finding that PGE2 levels and the activities of SOD and catalase are increased in the GB muscle that results from the decreased muscle response to CCK-8 and ACh that are involved in the neurohumoral regulation of GB emptying. This conserved PGE2 action may increase the GB tonic contraction and may reduce the entry of highly toxic hydrophobic bile acids (19, 42, 45). The mechanisms whereby PGE2 receptors and their functions are conserved are not fully understood (23, 24, 39). It is conceivable that the preservation of these receptors is the result of increased endogenous production of PGE2 (43). Ligands are known to protect their receptors against the actions of ROS (44).

Thus, the functional integrity of PGE2 receptors and their signal transduction pathways may play a cytoprotective role against ROS and hydrophobic bile salts. The preservation of the PGE2-induced contraction suggests that the increased production of eicosanoids may protect their membrane receptors (11). The mechanism involved in muscle cytoprotection appears to be mediated by PGE2 and other prostaglandins, since they stimulate the activities of scavengers of free radicals (4, 12, 16, 21, 22, 29, 43). The finding that PGE2 levels and the activities of SOD and catalase are increased in the GB muscle in acute cholecystitis and worsened by TCDC (42, 45) is consistent with the hypothesis that it may be induced by hydrophobic bile acids through the generation of H2O2 (45). These conclusions are also supported by the ability of PGE2 to prevent the development of oxidative stress when muscle cells are pretreated with this prostaglandin before their exposure to H2O2 and TCDC. Under these experimental conditions, the cells do not show the expected increase in lipid peroxidation and in the activities of scavengers of free radicals. The absence of an increase in scavengers of free radicals after the initial exposure of muscle cells to PGE2 followed by oxidative stress is probably the result of the consumption of scavengers as they inactivate ROS.

The muscle response to H2O2 and TCDC is significantly affected after muscle cells are treated with Ch-rich liposomes that increase the Ch levels in plasma membranes (47). The catalase activity was also reduced in response to H2O2 and TCDC and to PGE2. These may explain, in part, the failure of this prostaglandin to protect muscle cells against H2O2 and TCDC under these experimental conditions and possibly in GB with lithogenic bile. This impaired response appears to be because of defective PGE2 receptor functions (38). Similar results were obtained in muscle cells from human GB with Ch stones whose plasma membranes contain high concentrations of Ch (38). Thus the impaired cytoprotection response to H2O2 and TCDC may be an additional contributing factor that may render these GB susceptible to develop acute cholecystitis.

In summary, muscle contraction in response to H2O2 and TCDC may be an initial step in the cytoprotective process against damage by bile constituents. PGE2 may reduce the muscle cell damage caused by H2O2 and by TCDC, as demonstrated by the reduction in lipid peroxidation. The protection of the PGE2 receptors and their signal transduction may be designed to conserve their cytoprotective functions against hydrophobic bile salts and H2O2. The defective response to PGE2 in muscle cells treated with Ch-rich liposomes may explain the high incidence of acute cholecystitis in GB with symptomatic Ch stones (36). PGE2 and other prostaglandins may also be capable of functioning continuously since they are resistant to agonist-induced desensitization.

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