Receptor tyrosine and MAP kinase are involved in effects of 
H₂O₂ on interstitial cells of Cajal in murine intestine

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Abstract

Hydrogen peroxide (H₂O₂) is involved in intestinal motility through changes of smooth muscle activity. However, there is no report as to the modulatory effects of H₂O₂ on interstitial cells of Cajal (ICC). We investigated the H₂O₂ effects and signal transductions to determine whether the intestinal motility can be modulated through ICC. We performed whole-cell patch clamp in cultured ICC from murine intestine and molecular analyses. H₂O₂ hyperpolarized the membrane and inhibited pacemaker currents. These effects were inhibited by glibenclamide, an inhibitor of ATP-sensitive K⁺ (KATP) channels. The free-radical scavenger catalase inhibited the H₂O₂-induced effects. MAFP and AACOCF₃ (a cytosolic phospholipase A₂ inhibitors) or SC-560 and NS-398 (a selective COX-1 and 2 inhibitor) or AH6809 (an EP₂ receptor antagonist) inhibited the H₂O₂-induced effects. PD98059 (a mitogen activated/ERK-activating protein kinase inhibitor) inhibited the H₂O₂-induced effects, though SB-203580 (a p38 MAPK inhibitor) or a JNK inhibitor did not affect. H₂O₂-induced effects could not be inhibited by LY-294002 (an inhibitor of PI 3-kinases), calphostin C (a protein kinase C inhibitor) or SQ-22536 (an adenylate cyclase inhibitor). Adenoviral infection analysis revealed H₂O₂ stimulated tyrosine kinase activity and AG 1478 (an antagonist of epidermal growth factor receptor tyrosine kinase) inhibited the H₂O₂-induced effects. These results suggest H₂O₂ can modulate ICC pacemaker activity and this occur by the activation of KATP channels through PG₂ production via receptor tyrosine kinase-dependent MAP kinase activation.

Keywords: hydrogen peroxide – interstitial cells of Cajal – pacemaker currents – cyclooxygenase – receptor tyrosine kinase – MAP kinase

Introduction

Intestinal inflammation either in human beings or in experimental animal models is associated with altered gastrointestinal motility [1–3]. The mechanisms of altered motility have been associated with morphological and functional changes in smooth muscle and enteric nerves [4–6]. Large numbers of polymorphonuclear leucocytes that generate reactive oxygen species (ROS) such as H₂O₂ are found in the mucosa and submucosa of the inflamed bowel disease, suggesting that H₂O₂ plays an integral role in the inflammatory process [7]. Several in vitro studies have reported that exposure to H₂O₂ alters gastrointestinal smooth muscle contractility. For example, H₂O₂ decreases sigmoid smooth muscle contractility in ulcerative colitis patients and the radical scavenger catalase prevents ulcerative colitis-induced reduction of muscle contractions [8, 9]. Exposure to H₂O₂ reduces the lower esophageal sphincter tone in human esophagitis and treatment with catalase can restore the lower esophageal tone to normal [10]. From above findings, it is suggested that H₂O₂ may be an important mediator causing dysmotility in intestinal inflammation.

The gastrointestinal smooth muscles show spontaneous mechanical contractions. These contractions are mediated by the generation of periodic membrane depolarization (slow waves). It is well known that interstitial cells of Cajal (ICC) are pacemaker cells that generate slow waves, which are initiated by spontaneous inward currents (pacemaker currents) [11–13], even if some reports suggested that gastrointestinal pacing was possible without...
ICC and other pacemaker cells may be involved in pacing activity [14, 15]. ICC are coupled to each other and to smooth muscle cells via gap junctions. ICC also express various receptors for receiving inhibitory and excitatory signals from the enteric nervous systems [16, 17]. Acetic acid-induced inflammation reduces the membrane potential and reduces the amplitude and duration of slow waves in colonic circular muscle cells, suggesting that ICC may involve in motility changes in the inflammatory process [18].

Despite the observation that H2O2 is involved in intestinal motility through the changes of smooth muscle contractility, ion channel activity and enteric neuronal mechanisms, there are no reports describing the modulatory effects of H2O2 on pacemaker activities of ICC. In the present study, we investigated the effects of H2O2 on pacemaker currents and signal transductions to determine whether the intestinal motility can be modulated by ROS through ICC in the murine intestine.

Materials and methods

Preparation of cells and tissues

Balb/C mice (8- to 13-day old) of either sex were anaesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca2+ -free Hank’s solution for 30 min and the cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/ml, trypsin inhibitor (Sigma), 2 mg/ml and ATP, 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 g/ml) and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca2+ -free Hank’s solution for 30 min and the cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/ml, trypsin inhibitor (Sigma), 2 mg/ml and ATP, 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in 35-mm culture dishes. The cells were then cultured at 37°C in a 95% O2–5% CO2 incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 mg/ml murine stem cell factor (SCF, Sigma).

Patch-clamp experiments

Cultures of cells contained single cells and networks of cells that had gross morphological properties similar to ICC in situ, including fusiform cell bodies, large, prominent nuclei with little perinuclear cytoplasm and multiple, thin processes extending from the nuclear region that were often interconnected with processes of neighbouring cells [19]. Recordings were made from ICC with the patch-clamp technique as soon as the network-like structures. Recordings were made from cells within networks that had morphologies similar to the cells that were immunopositive for c-Kit.

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Currents or potentials were amplified by use of an Axopatch 1-D (Axon Instruments, Foster City, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on a computer monitor, and a pen recorder (Gould 2200, Gould, Valley view, OH, USA). All experiments were performed at 30°C.

Results

Effects of H2O2 on the pacemaker activity in ICC

To determine whether H2O2 have function on pacemaker activities of ICC, we recorded the pacemaker potential in a current clamp...
and pacemaker currents in a voltage clamp. Under the current clamp mode, H$_2$O$_2$ (1 mM) produced membrane hyperpolarization and decreased the amplitude of the pacemaker potential (Fig. 1A). Under control conditions at $I_{H11005}$, the resting membrane potential was $-52 \pm 4.8$ mV, and the amplitude of the pacemaker potential was $39.6 \pm 5$ mV. In the presence of H$_2$O$_2$, the membrane was hyperpolarized to $-76.3 \pm 8.0$ mV and the amplitude of the pacemaker potentials decreased to $7.6 \pm 3.9$ mV ($n = 6$, data not shown). Under a voltage clamp at a holding potential of $70$ mV, the ICC generated spontaneous inward currents. The mean frequency of these pacemaker currents was $13.9 \pm 1.4$ cycles/min and their mean amplitude and mean resting current levels were $416 \pm 42$ pA and $-26 \pm 7$ pA, respectively ($n = 7$). The addition of 10 $\mu$M H$_2$O$_2$ slightly reduced the amplitude and frequency of pacemaker currents and slightly increased resting currents in the outward direction (Fig. 1B). In the presence of 100 $\mu$M and 1 mM H$_2$O$_2$, the pacemaker currents were largely inhibited and the resting currents were increased in the outward direction (Fig. 1C and D). The inhibitory frequencies and amplitudes with H$_2$O$_2$ treatment were $5.1 \pm 1.8$ cycles/min and $-90.9 \pm 16$ pA at a concentration of 100 $\mu$M H$_2$O$_2$ and $2.8 \pm 1.4$ cycles/min and $-24 \pm 19$ pA at a concentration of 1 mM H$_2$O$_2$, respectively. The resting current levels were $23 \pm 5.9$ pA at a concentration of 100 $\mu$M H$_2$O$_2$ and $24.5 \pm 7.2$ pA at a concentration of 1 mM H$_2$O$_2$ ($n = 7$) (Fig. 1E–G). Next, to determine whether H$_2$O$_2$ affects K$_{ATP}$ channels in ICC, we used the K$_{ATP}$ channels inhibitor glibenclamide, after or before H$_2$O$_2$ treatment. The H$_2$O$_2$-induced effects on pacemaker currents were inhibited by co- or pretreatment with glibenclamide (Fig. 2A and E), indicating that H$_2$O$_2$ may activate K$_{ATP}$ channels in ICC. The results of glibenclamide treatment on the H$_2$O$_2$-induced effects on pacemaker currents are summarized in Figure 2B–D.

Effects of catalase and phospholipase A$2$ inhibitors on the H$_2$O$_2$-induced inhibition of pacemaker currents

To evaluate whether the H$_2$O$_2$-induced inhibition of pacemaker currents was mediated via a ROS and PLA$2$ signal pathway, we treated the ICC with catalase (3000 unit/ml), MAPF (10 $\mu$M) and AACOCF$_3$ (10 $\mu$M) before exposure to H$_2$O$_2$. Catalase inhibited the H$_2$O$_2$-induced effects (Fig. 3A) indicating that H$_2$O$_2$ may activate K$_{ATP}$ channels in ICC. The results of catalase treatment on the H$_2$O$_2$-induced effects on pacemaker currents are summarized in Figure 3B and C. As shown in Figure 3C–E, the values of the frequency, amplitude and resting...
currents by H2O2 in the presence of catalase, MAFP and AACOCF3 were significantly different from those obtained in the absence of catalase, MAFP and AACOCF3 (n = 5).

Involvement of cyclooxygenase and the prostaglandin E2 receptor in the H2O2-induced inhibition of pacemaker currents

Since activation of the PLA2 pathway would stimulate the cyclooxygenase (COX) and prostaglandins synthesis, we examined the role of this system in mediating the H2O2-induced effects using SC-560 (10 μM), a specific COX-1 inhibitor, or NS-398 (10 μM), a specific COX-2 inhibitor. ICC were pretreated with SC-560 (10 μM) or NS-398 (10 μM) prior to the treatment with H2O2. We found that the H2O2-induced effects was inhibited by pretreatment with SC-560 or NS-398 (n = 7, Fig. 4A and B). Our previous report suggested that PGE2-activated KATP channels through PGE2-Ep2 receptor activation in ICC [20]. Therefore, we tested AH6809 (10 μM), a PGE2-Ep2 receptor antagonist and found the H2O2-induced effects were inhibited by pretreatment with AH6809 (Fig. 5A). Furthermore, AH6809 inhibited the PGE2-induced effects (Fig. 5B). The values of the frequency, amplitude and resting currents induced by H2O2 in the presence of AH6809 were significantly different from those obtained in the absence of AH-6809 (n = 6, Fig. 5C–E). These results suggest that PGE2 may be involved in the H2O2-induced inhibition of pacemaker currents.

Involvement of mitogen-activated protein kinases (MAPKs) in the H2O2-induced inhibition of pacemaker currents

Since many reports suggested H2O2 activate MAPKs in many cell types, we investigated whether MAPKs are involved in the H2O2-induced effects using PD98059, a p44/42 MAPK inhibitor, or SB203580, a p38 MAPK inhibitor, or a JNK (c-jun NH2-terminal kinase) inhibitor. Figure 6A shows that PD98059 (10 μM) prevented the H2O2-induced effects. However, SB203580 and JNK inhibitor did not affect the H2O2-induced effects (Fig. 6B and C). The values of the frequency, amplitude and resting currents by H2O2 in the presence of PD98059 were significantly different from those obtained in the absence of PD98059 (n = 6, Fig. 6E–G). In
addition, PD98059 inhibited the PGE2-induced effects; supporting the role of PGE2 as a mediator of H2O2 (Fig. 6D).

**Effects of the adenylate cyclase inhibitor, PI3-kinase inhibitor and protein kinase C inhibitor in the H2O2-induced inhibition of pacemaker currents**

To determine an upstream regulator of p44/42 activation, ICC were pretreated with either 10 μM SQ-22536 (an adenylate cyclase inhibitor), 10 μM LY-294002 (a PI3-kinase inhibitor) and 0.1 μM calphostin C (a PKC inhibitor). We found that all inhibitors did not affect the H2O2-induced effects (n = 5; bar graph not shown), indicating that the H2O2-induced effects in ICC are part of a cAMP-, PI3-kinase- and PKC-independent pathway (Fig. 7A–C).

**Involvement of EGFR (epidermal growth factor receptor) tyrosine kinase in the H2O2-induced inhibition of pacemaker currents**

To evaluate the role of tyrosine kinase in the H2O2-induced effects, ICC were infected with adenovirus that was constructed containing a tyrosine kinase gene. In Figure 7D, we could see the tyrosine kinase domain was located predominantly in the nucleus of ICC. When ICC were treated with H2O2 (1 mM) for 30 min, there was a translocation of the tyrosine kinase from nucleus to the cytosol (Fig. 7E). This finding indicates that H2O2 stimulates the tyrosine kinase activity in ICC. Furthermore, to determine whether the H2O2-induced inhibition of pacemaker currents is mediated by EGFR tyrosine kinase, we treated the cells with AG 1478, a potent and selective inhibitor of EGFR tyrosine kinase. AG 1478 (10 μM) inhibited the H2O2-induced effects (Fig. 7F). Also, we found that AG 1478 inhibited the PGE2-induced inhibition of pacemaker currents.
Fig. 4. The effect of SC-560, a specific cyclooxygenase-1 inhibitor, and NS-398, a specific cyclooxygenase-2 inhibitor, on the H$_2$O$_2$-induced effects on pacemaker currents of ICC from the murine intestine. (A) Pacemaker currents exposed to 1 mM H$_2$O$_2$ after pretreating cells with 10 µM SC-560 at a holding potential of $-70$ mV. (B) The effect of 1 mM H$_2$O$_2$ on pacemaker currents after pretreating cells with 10 µM NS-398. (C, D, and E) The inhibitory response to SC-560 or NS-398 on the H$_2$O$_2$-induced action on pacemaker currents. The bars represent mean ± S.E. values ($n = 7$). Asterisks indicate significantly different from H$_2$O$_2$ alone ($P < 0.05$).

Fig. 5. The effect of AH6809, a PGE$_2$-EP$_2$ receptor antagonist, on the H$_2$O$_2$-induced effects on pacemaker currents of ICC from the murine intestine. (A) The effect of 1 mM H$_2$O$_2$ on pacemaker currents after pretreating cells with 10 µM AH6809. (B) The effect of 1 µM PGE$_2$ on pacemaker currents after pretreating cells with 10 µM AH6809. (C, D, and E) The inhibitory response to AH6809 on the H$_2$O$_2$-induced effects on pacemaker currents. The bars represent mean ± S.E. values ($n = 6$). Asterisks indicate significantly different from H$_2$O$_2$ alone ($P < 0.05$).
Fig. 6. The effect of PD98059, SB203580, and the JNK inhibitor on the H2O2-induced effects on pacemaker currents of ICC from the murine intestine. (A) The effect of 1 mM H2O2 on pacemaker currents after pretreating cells with 10 μM PD98059, a p44/42 MAPK inhibitor, for 15 min. (B) The effect of 1 mM H2O2 on pacemaker currents after pretreating cells with 10 μM SB203580, a p38 MAPK inhibitor, for 15 min. (C) The effect of 1 mM H2O2 on pacemaker currents after pretreating cells with 10 μM JNK inhibitor, c-jun NH2-terminal kinase inhibitor, for 15 min. (D) The effect of 1 μM PGE2 on pacemaker currents after pretreating cells with 10 μM PD98059 for 15 min. (E, F, and G) The inhibitory response to PD98059 on the H2O2-induced action on pacemaker currents. The bars represent mean ± S.E. values (n = 6/group). Asterisks indicate significantly different from H2O2 alone (P < 0.05), and the dotted lines indicate zero current levels. PD98059, PD; SB203580, SB; JNK inhibitor, JNK.

Discussion

In the present study, we first demonstrated that H2O2 induces hyperpolarization of the membrane and inhibits pacemaker currents in intestinal ICC. These effects are mediated through activation of KATP channels by COX-dependent PGE2 production, and receptor tyrosine and MAP kinase are involved in the H2O2-induced process.

K+ channels play an important role in regulating cellular excitability in various cell types. H2O2 hyperpolarizes the resting membrane potential through the activation of several K+ channels in vascular and visceral smooth muscles [21, 22]. The activation of KATP channels would lead to membrane hyperpolarization, which is thought to be an important mechanism for smooth muscle relaxation. We have already functionally reported that ICC have an KATP channels and that the ICC are modulated by bile salts and antidepressants [23, 24]. Deoxycholic acid inhibited pacemaker currents that were blocked by glibenclamide, an inhibitor of the KATP channel, and imipramine blocked the pinacidil (an opener of the KATP channels)-induced inhibition in a similar manner as glibenclamide. KATP channels is comprised of Kir 6.2 with SUR2B in cultured ICC of the mouse [20], indicating these channels may play an important role in the electrical activities of ICC and be a target of endogenous substances and drugs, as in smooth muscle. In the present study, H2O2 hyperpolarized the membrane potential and inhibited pacemaker currents. These effects were inhibited by glibenclamide, which indicates that H2O2 can change pacemaker activities through the activation of KATP channels in ICC.

H2O2 mediates the production of PGs from arachidonic acid via COX enzyme activation in GI tract. For examples, exposure to H2O2 causes damage to the plasma membrane of the gallbladder muscle and contraction through the generation of PGE2 by the cPLA2-cyclooxygenase pathway [25]. H2O2 reduces the lower esophageal sphincter (LES) tone in human esophagitis and the cat LES tone by increasing the synthesis of COX-2 and PGE2 [10, 26]. And we reported that deoxycholic acid activated COX-2-dependent PGE2 production [23]. The biological effects of PGE2 are mediated via four different receptor subtypes (EP1, -2, -3 and -4) [27]. We found that PGE2 action on pacemaker currents in ICC was mediated by EP2 receptors and the expression of the EP2 subtype was only detected [20]. In the present study, H2O2-induced effects were blocked by cPLA2 inhibitors (MAFP and AACOCF3) or COX inhibitors (SC560 and NS-398) as well as by an EP2-receptor antagonist (AH6809). Taken together, these results suggest that the inhibition of pacemaker currents through activation of KATP channels by H2O2 is mediated by cPLA2-COX-dependent PGE2 production.

A remaining issue to consider is the determination of the signal linkage between production of PGE2 by H2O2 and the activation of KATP channels. The MAPKs signaling pathway plays an important role in the mediation of cellular responses including visceral smooth muscle contraction [28]. Three principal
MAPKs are expressed in various tissues: p44/42 MAPK, JNK and p38 MAPK [29]. H₂O₂ and PGE₂ activate MAPKs in many cell types [30–32]. In the present study, PD98059, an inhibitor of p44/42 MAPK, inhibited the H₂O₂- or PGE₂-induced inhibition of pacemaker currents suggesting p44/42 MAPK may be involved in the modulation of pacemaker currents by H₂O₂. It has been reported that H₂O₂ induced ERK1/2 activation in cultured feline ileal smooth muscle cells and pulmonary arterial smooth muscle cells [32, 33]. In addition, MAPK-ERK is involved in the activation of K<sub>ATP</sub> channel by nitric oxide in neurons [34]. In addition, PGE₂ activates ERK1/2 in several cells [35, 36], which supports our results.

cAMP, PI3-kinase, PKC and tyrosine kinase are upstream regulators in MAPKs activation [22, 37]. The cAMP pathway is the major inhibitory mechanism responsible for the relaxation of gastrointestinal smooth muscle [38]. In addition, K<sub>ATP</sub> channels are opened by the cAMP-dependent signal pathway in smooth muscle cells [39]. However, in the current study, treatment with an adenylate cyclase inhibitor had no influence on the H₂O₂-induced effects. Also, cell permeable 8-bromo-cAMP did not inhibit pacemaker currents [40], indicating cAMP is not involved in the H₂O₂-induced effects. An antagonist of PI3-kinase, LY-294002 suppresses H₂O₂-induced contraction in vascular smooth muscle cells [41]. PI3-kinase also has been reported to mediate ERK1/2 activation by insulin and thrombin [42, 43]. PI3 kinase leads to activation of PKC [44]. In addition, H₂O₂ has been reported to stimulate PKC activities [45]. Also, some reports suggested that spontaneous rhythmic contractions of uterus and intestine were inhibited by a novel and potent inhibitor of c-Kit tyrosine kinase [46, 47]. In this study, LY-294002 or PKC blocker (calphostin C) did not affect H₂O₂-induced effects. Generally PKC activation produces smooth muscle contraction. Therefore, these results suggest that cAMP, PI3-kinase and PKC are not involved in the H₂O₂-signal pathways, indicating that other mechanisms may be involved in the activation of K<sub>ATP</sub> channels in intestinal ICC by H₂O₂.

H₂O₂ increases tyrosine phosphorylation of proteins in different cell types [32]. Receptor tyrosine kinase, such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and insulin-like growth factor (IGFR), have been
demonstrated to be targets of ROS [48, 49]. It has been reported that H2O2-induced p44/42 activation was mediated by the receptor tyrosine kinase of EGFR in feline ileal smooth muscle cells and vascular smooth muscle cells [32, 33]. Based on these findings, we examined the activation of EGFR using an EGFR antagonist (AG1478) before exposure to H2O2. We found that EGFR antagonist inhibited the H2O2-induced effects. It has been reported that PGE2 tyrosine kinase inhibitor inhibited PGE2-induced effects. Taken together these findings suggest that the activation of EGFR by H2O2 via PGE2 may involve the regulation of pacemaker currents.

In conclusion, we have demonstrated that the inhibition of pacemaker currents by H2O2 in cultured ICC has mediated the activation of COX, with a consequent increase of PGE2 production. And we have showed that during these events, the increasing of PGE2 can influence on KATP channels, the receptor tyrosine kinase of EGFR and also MAPK (p44/42) pathway.

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