Evidence for $P_2$-purinoceptors contribution in $H_2O_2$-induced contraction of rat aorta in the absence of endothelium

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Abstract

Objective: $H_2O_2$ can contract many arteries, however the underlying mechanisms are not fully understood. This study aims to test whether $H_2O_2$-induced vasoconstriction could be functionally attributed to the activation of $P_2$-purinoceptors in rat aorta and to explore its possible signaling mechanisms. Methods: Isometric tension recording of $H_2O_2$ and ATP-induced contractions of rat aortic rings were compared in the absence or presence of various pharmacological tools to identify their possible common signaling pathways. Results: Both $H_2O_2$ and ATP induced transient phasic contractions in a concentration-dependent manner (1–1000 μM). Removal of endothelium potentiated the contractile responses to $H_2O_2$ and to ATP. $H_2O_2$ (30 μM)-induced phasic contraction could be abolished by catalase (800 U/ml), but not affected by SOD (150 U/ml), DMSO (5 mM) and apyrase (5 U/ml), suggesting no involvement of $O_2^••$, hydroxyl free radicals and ATP release. Also, several receptor antagonists including phentolamine, atropine, methysergide and chlorpheniramine (each 3 μM) were without effect on $H_2O_2$ (30 μM)-induced phasic contraction, suggesting no involvement of typical neurotransmitter release. However, both $H_2O_2$ (30 μM) and ATP (1 mM)-induced phasic contractions not only presented homologous desensitization, but also showed heterogeneous desensitization. Furthermore, the phasic contractions in response to $H_2O_2$ (30 μM) or ATP (100 μM) could be inhibited or abolished in a concentration dependent manner by RB-2 and suramin (10–100 μM), two widely used $P_2$-purinoceptor antagonists, with only partial inhibition by Evans blue (300 μM), a moderately selective $P_2$ receptor blocker, or by α-β-methylene-ATP (100 μM), a selective $P_2$ receptor desensitizer. On the other hand, both $H_2O_2$ (30 μM) and ATP (100 μM)-induced phasic contractions were also attenuated, to different degree, by inhibitors of several enzymes including PLC, PKC, PLA$_2$, and cyclooxygenase. Lastly, removal of extracellular Ca$^{2+}$ or pretreatment with procaine (10 mM) and dantrolene (30 μM), two putative intracellular Ca$^{2+}$ release blockers, or with Ni$^{2+}$ (100 μM) and tetrandrine (5 μM), two Ca$^{2+}$ channel blockers, all significantly inhibited $H_2O_2$ and ATP-induced contractions. However, nifedipine (1 μM), a voltage-dependent L-type Ca$^{2+}$ channel blocker, was without effect. Conclusions: Our results demonstrate that $H_2O_2$-induced phasic contraction of rat aorta involves, at least in part, the activation of $P_2$-purinoceptors in the aortic smooth muscle cells © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Arteries; Endothelial receptors; Free radicals; Smooth muscle; Vasoconstriction/dilation

1. Introduction

It has been demonstrated in recent years, that hydrogen peroxide (H$_2$O$_2$), like NO, acts as either a cellular signaling molecule or a toxic substance depending on its source and concentration [1,2]. Intracellular production of H$_2$O$_2$ can be triggered by various extracellular stimuli including G-protein coupled or uncoupled receptors activation [3,4]. However, the major source of H$_2$O$_2$ may come from its secretion by activated neutrophils or monocytes. In this case, the local concentration of H$_2$O$_2$ in vasculature could be up to millimolar levels [5]. Thus, the regulatory effect of H$_2$O$_2$ on vascular muscle tone has been intensive-ly studied. Indeed, previous studies had shown that H$_2$O$_2$
can cause contraction of many arteries in vitro, such as rat and rabbit aorta [6,7], canine coronary artery [8], rat and bovine pulmonary artery [9,10], canine basilar artery [11] and human placental arteries [12]. Furthermore, several potential mechanisms underlying $H_2O_2$-induced contraction have been proposed, including perturbation of $Ca^{2+}$ regulatory mechanisms, such as intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx in smooth muscle [13] or activation of several enzymes such as phospholipase A$_2$ (PLA$_2$) [14], phospholipase C (PLC) [15], protein kinase C (PKC) [13], cyclooxygenase (COX) [6] and tyrosine kinase [16]. Nevertheless, up to now, a possible unifying or a primary signaling step which could explain such a contractile effect induced by $H_2O_2$ is still missing.

Considering the fact that activation of PLC, PLA$_2$, or tyrosine kinase, in most cases, is the subsequent signaling steps resulting from the activation of G-protein coupled or uncoupled receptors and the previous suggestion that $H_2O_2$ mobilizes $Ca^{2+}$ in both smooth muscle and endothelial cells via a receptor-sensitive pathway [17–19], we, therefore, hypothesize that $H_2O_2$-induced vasoconstriction, particularly at low concentrations, may be due to its direct or indirect effect on some plasmalemmal receptors, rather than a highly non-specific effect as generally believed for many years. In this study, we focused on the $P_2$-purinoceptors, since several lines of evidence raised such a possibility. First, it was reported that $H_2O_2$-activated MAPKs signaling cascades in vascular smooth muscle cells could be inhibited by a non-selective $P_2$-purinoceptor antagonist suramin [21]; second, Musat et al. [21] have shown that ATP binding to cardiac plasmalemma ATP receptors (presumably $P_2$-purinoceptors) can be concentration- and time-dependently modulated by $H_2O_2$, suggesting a possible modification of $P_2$-purinoceptors by $H_2O_2$. Lastly, a recent study by Wartenberg et al. [22] indicated that in a human prostate cancer cell line, both $H_2O_2$ and ATP, via similar mechanisms, triggered either proliferative or growth inhibition effects depending on their testing concentrations. In view of these findings, the present study was designed to test whether $H_2O_2$-induced vasoconstriction could be functionally attributed to the activation of $P_2$-purinoceptors.

It is well known that $P_2$-purinoceptors are further classified into a series of $P_2x$ and $P_2y$ subtypes, which are based on their agonist selectivity. The fast transmitter function of ATP, including its vasoconstrictor function, is mediated by $P_2x$ receptors, which are part of ligand-gated ion channels and present in vascular smooth muscle cells. The $P_2y$ receptors are highly expressed in both endothelial cells and vascular smooth muscle cells and are linked to various second messenger systems including PLC, PLA$_2$, PKC and MAPKs via coupling of G-proteins [34,35,41]. In this work, we provided functional evidence to suggest that $P_2$-purinoceptors are involved in $H_2O_2$-induced contraction of rat aorta, and this effect, more importantly, cannot be ascribed to possible ATP release due to $H_2O_2$ challenge.

### 2. Methods

#### 2.1. Tissue-bath experiments

For this work, 3-month-old Male Sprague–Dawlay rats, weighing 250–300 g were used. Rats were maintained at the University facility for experimental animals under standard conditions and conformed to rules set by Animal Ethic Committee. The rats were killed by cervical dislocation under ether anesthesia. Rat aortas were isolated, and excess fat and connective tissues were removed. Vessels were cut into rings of about 3 mm width. The aortic ring was mounted vertically in a 3-ml organ bath, connected to a force transducer and a pen-recorder. The organ baths contain Krebs’ solutions with the following composition (mM): NaCl, 118; KCl, 4.7; CaCl$_2$, 2.5; MgCl$_2$-6 $H_2O$, 1.18; $KH_2PO_4$, 1.08; $NaHCO_3$, 25; and glucose, 11 at pH 7.4, maintained at 37°C and bubbled with a 95% $O_2$–5% $CO_2$ gas mixture. $Ca^{2+}$-free medium was prepared by omitting $CaCl_2$ from the normal Krebs’ solution and replaced with EGTA 100 $\mu$M. The solution in the baths was changed every 15 min. The rings were equilibrated for 20 min before stretching them to ~2 g and were allowed to further equilibrate for 90 min. Before data collection, stimulation of the rings with 50 mM KCl was repeated every 20 min until a reproducible contractile response was obtained.

#### 2.2. Experimental

##### 2.2.1. Specimen preparation

To preclude the possible impact of endothelium on the vascular effect induced by $H_2O_2$, most of the tests were conducted in endothelium-denuded preparations. So the endothelium was intentionally removed by gently rubbing against the teeth of a pair of forceps. The successful removal of endothelium was assessed by showing that acetylcholine 1 $\mu$M failed to relax the rings precontracted with phenylephrine 1 $\mu$M.

##### 2.2.2. Preliminary study

In most cases, only one dose of $H_2O_2$ or ATP was applied to the resting ring to observe their vasoconstrictr response, since both $H_2O_2$ and ATP-induced contractions showed tachyphlaxis phenomenon. Therefore, a cumulative concentration–response curve could not be constructed. To determine whether $H_2O_2$-induced contraction as related to endogenous vasoconstrictor release, or the formation of other free radicals, ring preparations were treated with various receptor antagonists for 20 min, or with SOD for 5 min, before addition of $H_2O_2$.

##### 2.2.3. Purinoceptor detection

To determine whether $P_2$-purinoceptors are involved in $H_2O_2$-induced contraction, aortic rings were pretreated with the putative $P_2$-purinoceptor antagonists suramin,
reactive blue 2 (RB-2) or Evans blue with different concentrations for 20 min, or pretreated with P2x receptors with α-β-methylene-ATP. Forces developed in response to H2O2 or ATP in the presence or absence of various blockers were compared.

2.2.4. Elucidating post-receptor mechanisms

The post-receptor signaling mechanisms of H2O2 and ATP-induced vasoconstriction, such as the involvement of PLC, PLA2 and PKC, were also investigated by pretreatment of aortic rings with a variety of enzyme inhibitors for 20 min. To test whether H2O2 and ATP-induced contractions are dependent on Ca2+ influx, rat aortic rings incubated in organ baths were washed three times in 5 min with Ca2+-free solution containing EGTA 100 μM [52], or pre-treated with different types of Ca2+ channel blockers for 20 min before addition of ATP or H2O2. To study whether intracellular Ca2+ release also participates in H2O2 and ATP-induced contractions, aortic rings were pretreated with intracellular Ca2+ release blockers before addition of H2O2 or ATP. The concentrations of all the pharmacological tools were used according to their values of IC50 in vascular smooth muscle. In some cases, the contractions induced by phenylephrine (1 μM), KCl (30 mM) or caffeine (10 mM) were also compared in the presence or absence of some blockers to confirm their inhibitory efficacy or selectivity on the aortic rings under our experimental conditions.

3. Results

3.1. Phasic contractions of rat aortic rings induced by H2O2 and ATP

Fig. 1 shows that in endothelium intact resting rings, both H2O2 and ATP elicited phasic contraction in a dose-related manner. The pattern of contraction induced by H2O2 was similar to that of ATP. Removal of endothelium potentiated the amplitude of phasic contraction in response to ATP or of H2O2. The time duration of the phasic contraction in response to H2O2 (1 mM) is about 10 min, and the maximal tension triggered by H2O2 (1 mM) is 606±77 mg. The same parameters for ATP (1 mM) are approximate 8 min and 482±54 mg. A concentration of H2O2 (30 μM) close to its EC50 was selected for further experiments. Table 1 shows that pretreatment of aortic rings with phentolamine, atropine, methysergide or chlorpheniramine (each 3 μM) did not significantly affect H2O2 (30 μM)-induced contraction.

3.2. Effects of SOD, DMSO, catalase and apyrase on H2O2- and ATP-induced contractions

Table 1 also shows that pretreatment of the aortic rings with SOD (150 U/ml) or DMSO (3 mM) did not affect H2O2 (30 μM)-induced contraction, indicating no involvement of superoxide anion (O2−) and hydroxyl free radicals. On the other hand, catalase (800 U/ml) pretreatment completely abolished H2O2 (30 μM)-induced contraction, with no effect on ATP (100 μM)-induced contraction. In contrast, pretreatment of the aortic rings with apyrase (5 U/ml), an ATP hydrolase, inhibited ATP (100 μM)-induced contraction by 90%, with no significant effect on H2O2 (30 μM)-induced contraction (Fig. 2A and B). These results clearly indicate that H2O2-induced contractions are independent on a possible release of ATP and ATP-induced contractions are not related to a possible release of H2O2.

3.3. Tachyphylaxis and cross-desensitization for H2O2- and ATP-induced contractions

After the first contraction induced by 30 μM H2O2, which was subsequently washed out for 30 min, a 2nd challenge of 30 μM H2O2 failed to induce a contraction,
Table 1  Effect of selected inhibitors on the contraction induced by 30 μM H₂O₂ in rat aorta

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Control tissues</th>
<th>n</th>
<th>Test tissues</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phentolamine (3 μM)</td>
<td>46.6±2.7</td>
<td>6</td>
<td>51.2±3.1</td>
<td>7</td>
</tr>
<tr>
<td>Atropine (3 μM)</td>
<td>33.8±3.0</td>
<td>5</td>
<td>37.3±2.4</td>
<td>5</td>
</tr>
<tr>
<td>Methysergide (3 μM)</td>
<td>48.3±4.1</td>
<td>5</td>
<td>46.8±3.9</td>
<td>6</td>
</tr>
<tr>
<td>Chlorpheniramine (3 μM)</td>
<td>40.4±2.7</td>
<td>7</td>
<td>43.6±2.8</td>
<td>7</td>
</tr>
<tr>
<td>DMSO (3 mM)</td>
<td>38.4±2.4</td>
<td>6</td>
<td>35.3±3.0</td>
<td>6</td>
</tr>
<tr>
<td>SOD (150 U/ml)</td>
<td>47.6±3.2</td>
<td>6</td>
<td>42.1±2.5</td>
<td>6</td>
</tr>
</tbody>
</table>

*Rat aortic rings denuded of endothelium were treated with inhibitors or vehicles for 20 min (SOD for 5 min) before addition of 30 μM H₂O₂. Data represent mean±S.E.M., expressed as percentage of the tension developed by 50 mM KCl and taken from at least three rats. No inhibition of H₂O₂-induced contraction was observed by each of the selected inhibitors.

Fig. 1. Typical trace showing the non-cumulative concentration-dependent phasic contraction induced by ATP (A) and H₂O₂ (B) in rat aortic rings with (E+) and without (E−) endothelium. Each ring preparation was challenged non-cumulatively with only one concentration of H₂O₂ or ATP as indicated (in μM). (C) Graph shows the representative concentration–response curves for ATP and H₂O₂; vertical lines indicate S.E.M. Data in each tracing are representative of experiments using five individual aortic rings taken from at least three rats, and expressed in mg of tension developed.

and totally abolished ATP (1 mM)-induced contraction. Similarly, when aortic rings were first contracted with 1 mM ATP followed by washout for 30 min, a 2nd application of 1 mM ATP triggered only a small transient contraction and also significantly reduced the contraction induced by 30 μM H₂O₂ (Fig. 3A and B). However, using the same protocols, 30 μM H₂O₂ or 1 mM ATP did not significantly affect the contraction induced by 30 mM KCl, 1 μM PE or 10 mM caffeine (Fig. 3C–E).

Fig. 2. Effects of catalase (800 U/ml) and apyrase (5 U/ml) on the contractions induced by 30 μM H₂O₂ (A) or 100 μM ATP (B) in endothelium-denuded rat aortic rings. Each preparation was challenged with H₂O₂ or ATP only once after preincubation with either vehicle (control), catalase or apyrase for 5 min. Each column represents the mean±S.E.M. (n=6–9), expressed as percentage of the tension developed by 50 mM KCl and taken from at least three rats. *, P<0.001 compared with control.
Fig. 3. Tachyphylaxis (A) and cross-desensitization (B) of contractile responses induced by 30 μM H₂O₂ and 1 mM ATP in endothelium-denuded rat aortic rings. Using the same protocols, H₂O₂ or ATP did not affect the contractions induced by 1 μM phenylepherine (PE, C), 30 mM KCl (D) or 10 mM caffeine (E). Test drugs were applied at the points indicated by squares. || denotes washout for 30 min. Tracings on the left-hand side and on the right-hand side represent different experiments using different aortic rings. Data in each tracing are representative of experiments using five individual aortic rings taken from at least three rats.
3.4. Effects of P₂-purinoceptor antagonists or desensitizer on H₂O₂ and ATP-induced contractions

Fig. 4A and B shows that RB-2 and suramin (10–100 μM), two widely used P₂-purinoceptor antagonists, concentration dependently inhibited H₂O₂ (30 μM)-induced contraction in endothelium-denuded preparations. Similar results were observed for 100 μM ATP (Fig. 4C and D). On the other hand, α-β-methylene-ATP (100 μM), a selective P₂x receptor agonist and desensitizer, also triggered a phasic contraction with a pattern similar to that induced by 100 μM ATP. In addition, after washout of α-β-methylene-ATP, a second challenge of aortic rings with the same concentration of α-β-methylene-ATP produced negligible contraction (Fig. 5A). However, α-β-methylene-ATP, under the same experimental protocol, only slightly depressed the contraction induced by 100 μM ATP or 30 μM H₂O₂ (Fig. 5B and C). Similarly, evans blue at 300 μM, a moderately selective P₂x receptor blocker, completely abolished α-β-methylene-ATP (100 μM)-induced contraction (Fig. 5A), with only slight inhibition of ATP (100 μM) and H₂O₂ (30 μM)-induced contractions (Fig. 5B and C). Neither suramin and RB-2 nor evans blue and α-β-methylene-ATP at concentrations up to 300 μM could affect KCl (30 mM) or PE (1 μM)-induced contractions in rat aortic rings (data not shown). Fig. 6A and B further shows that although preincubation of the rat aortic rings with suramin (100 μM) abolished ATP (100 μM)- and H₂O₂ (30 μM)-induced contractions, after washout of suramin, ATP and H₂O₂ could still elicit phasic contractions with amplitude comparable to that of controls. However, after pre-incubation with RB-2 (100 μM), both ATP- and H₂O₂-induced contractions were irreversibly blocked (Fig. 6C and D). This result is consistent with the notion that suramin is a reversible and RB-2 is an irreversible antagonist of P₂-purinoceptors.

3.5. Roles of PLC, PLA₂, COX and PKC in H₂O₂ and ATP-induced contractions

In normal Krebs’ solutions, H₂O₂ (30 μM)-induced contractions...
contractions were significantly depressed by 10 μM NCDC, a PLC inhibitor [23]; by 5 mM neomycin, a chelator of plasmalemmal PIP2 [24]; by 10 μM H7, a wide-spectrum inhibitor of protein kinases including PKC [25]; by 50 μM mepacrine, a PLA2 inhibitor [26]; or by 3 μM indomethacin, a COX inhibitor (Fig. 7A). The same concentration of NCDC, neomycin, H7, mepacrine and indomethacin also blocked ATP (100 μM)-induced contraction (Fig. 7B), with no influence on KCl (30 mM)-induced contraction (data not shown).

3.6. Effects of Ca²⁺ removal, procaína, dantrolene, Nif²⁺, nifedipine and tetrandrine on H₂O₂- and ATP-induced contractions

After pretreatment of the aortic rings with Ca²⁺-free Krebs’ solution (see Experimental), 30 μM H₂O₂ still triggered a similar phasic contraction with a smaller magnitude compared to that obtained in Ca²⁺-containing solution (Fig. 8A). Similar results were obtained when preparations were pretreated with 10 mM procaína and 30
μM dantrolene, two putative intracellular Ca\(^{2+}\) release blockers [27,28]. On the other hand, the contractions induced by 30 μM H\(_2\)O\(_2\) were nearly abolished by 100 μM Ni\(^{2+}\), a non-selective inorganic Ca\(^{2+}\) channel blocker, and also largely inhibited by 30 μM tetrandrine, an organic blocker of Ca\(^{2+}\) channel blocker (for review, see [29,30]). In contrast, 1 μM nifedipine which could inhibit KCl-induced contraction, had no effect on the contraction induced by 30 μM H\(_2\)O\(_2\) (Fig. 8A). Comparable inhibitory effects of the various blockers except for nifedipine on the contraction induced by 100 μM ATP were also observed (Fig. 8B).

4. Discussion

4.1. H\(_2\)O\(_2\)-induced vasoconstriction is pharmacologically selective

In the present study, we found H\(_2\)O\(_2\), a mild endogenous oxidant, can induce an apparent phasic contraction of rat aorta even at a physiological concentration of 30 μM [31]. Such a transient contraction had been reported previously in rat pulmonary arteries [16] and more recently in aorta of WKY and SHR rats [6]. H\(_2\)O\(_2\)-induced contraction is not
related to the release of typical neurotransmitters, because the response to H$_2$O$_2$ was not affected by phenolamine, an α-adrenoceptor antagonist; atropine, a muscarinic acetylcholine receptor antagonist; methysergide, a serotonin-receptor antagonist and chlorpheniramine, a histamine-receptor antagonist. In fact, it is widely accepted that rat aorta is not sympathetically innervated. However, as anticipated, catalase pretreatment could abolish H$_2$O$_2$-induced phasic contraction, indicating that the response is truly induced by H$_2$O$_2$. However, superoxide anion (O$_2^-$) and hydroxyl free radicals may not be involved, because the O$_2^-$ scavenger, SOD and the hydroxyl free radical scavenger, DMSO, failed to inhibit H$_2$O$_2$-induced contraction.

It is interesting to note that H$_2$O$_2$-induced contraction is similar to ATP-induced contraction, and that both H$_2$O$_2$- and ATP-induced contractions were potentiated by endothelium removal. These results suggest that both H$_2$O$_2$ and ATP may trigger the release of endothelium-derived vasodilators, presumably as NO and/or PGL$_2$, which may compromise H$_2$O$_2$ and ATP-induced contractions [6,32]. Furthermore, Musat et al. [21] had shown that H$_2$O$_2$ could, in a concentration- and time-dependent manner, modulate ATP binding to plasmalemma ATP receptors (presumably including P$_2$-purinoceptors), suggesting a possible direct modification of P$_2$-purinoceptors by H$_2$O$_2$. The interaction between H$_2$O$_2$ and the ATP-binding site, presumably the P$_2$-purinoceptors is further supported by the finding that H$_2$O$_2$-activated MAPKs signaling cascades in aortic smooth muscle cells could be blocked by suramin, a non-selective antagonist of P$_2$-purinoceptors [20]. Based on these observations, we speculate that H$_2$O$_2$ may contract rat aortic rings via activation of P$_2$-purinoceptors. However, in view of the findings that stress on cells such as mechanical stretch, e.g. as in cell swelling [33] or hypoxia [34] can cause the cell to release ATP, it is possible that our observed contractile responses to H$_2$O$_2$ might be due to the autocrine action of ATP brought about by H$_2$O$_2$. In addition, the lack of inhibitory effect of phentolamine and the poor sympathetic innervation in rat aorta makes the possibility of H$_2$O$_2$-induced release of ATP as a co-transmitter with norepinephrine from the nerve endings highly unlikely. Furthermore, the fact that apyrase, at a concentration that nearly abolished ATP-induced contraction, had no influence on H$_2$O$_2$-induced contraction, would also argue against the pathway due to ATP release.

4.2. H$_2$O$_2$ directly activates P$_2$-purinoceptors

The involvement of P$_2$-purinoceptors activation in response to H$_2$O$_2$ was further assessed. We observed that H$_2$O$_2$ and ATP-induced contractions not only showed homologous functional desensitization, but also heterologous desensitization against each other. Under the same experimental protocols, however, H$_2$O$_2$ and ATP had no significant effect on KCl, PE or caffeine-induced contractions, indicating that brief treatment (10 min) of rat aorta with low dose of H$_2$O$_2$ (30 μM) or high concentration of ATP (1 mM) would not non-selectively impair smooth muscle contractions. This observation is consistent with an early finding by Jin et al. [16] using rat pulmonary arteries. Therefore, the results of cross-desensitization tests lend additional support to our contention that H$_2$O$_2$ and ATP may share common target(s), mediating their contractile responses in rat aorta.

The fact that both suramin and RB-2, two widely used P$_2$-purinoceptor antagonists [35], could concentration-dependently inhibit or abolish H$_2$O$_2$- or ATP-induced contractions, further confirming that P$_2$-purinoceptors activation participates in H$_2$O$_2$-induced contraction of rat aorta. Since P$_2$-purinoceptors can be further classified as P$_{2X}$ and P$_{2Y}$ subtypes [35], we further tested which subtype of P$_2$-purinoceptors mediates H$_2$O$_2$-induced phasic contraction. Unlike suramin and RB-2, evans blue which was demonstrated recently to be a moderately selective P$_{2X}$ receptor blocker [35,36], could abolish the selective P$_{2X}$ receptor agonist α-β-methylene-ATP-induced contraction of rat aorta, with only partial inhibition of ATP or H$_2$O$_2$-induced contractions, suggesting possible involvement of both P$_{2X}$ and P$_{2Y}$ receptors in ATP and H$_2$O$_2$ responses. Such a view is further supported by the finding that both ATP- and H$_2$O$_2$-induced contractions are only partially depressed when the preparations are subject to prior desensitization of P$_{2X}$ receptors with α-β-methylene-ATP. The fact that suramin, which does not readily cross cell membranes due to its large size and highly polar nature [35], could blunt the vascular contractile response induced by H$_2$O$_2$, suggests that despite easy diffusion of exogenous H$_2$O$_2$ to the cell interior, the contractile action of H$_2$O$_2$, at least at the concentration of 30 μM, may be primarily due to its direct effects on the surface membrane. The lack of effect of H$_2$O$_2$ on KCl, and PE-induced contractions also supports the view that H$_2$O$_2$ at concentration of 30 μM, does not elicit non-selective effects on cellular membrane or contractile elements. Although the nature of interaction between H$_2$O$_2$ and P$_2$-purinoceptors is unclear, the fact of reversible inhibition by suramin and irreversible inhibition by RB-2 on H$_2$O$_2$-induced contraction raises the possibility that suramin and RB-2 may have a ‘sealing effect’ on the H$_2$O$_2$-modified sites locating at P$_2$-purinoceptors and possible other plasmalemma proteins. Further study is required to elucidate this question.

4.3. H$_2$O$_2$ and ATP signaling pathways are similar

We also compared the signal transduction pathways which led to contraction in response to H$_2$O$_2$ and ATP. An increase in the production of IP$_3$ by the activation of PLC via P$_{2Y}$ receptors has been demonstrated in various tissues, including vascular smooth muscle [34]. NCDC has been shown to inhibit the activity of phosphoinositide-specific PLC [23], and also to inhibit the contractile responses induced by several P$_{2Y}$ receptor agonists in rat urinary bladder smooth muscle [37]. Therefore, in this study, we
used NCDC to inhibit the activity of PLC in rat aortic smooth muscle cells. At concentration of 10 \( \mu \text{M} \), NCDC inhibited contraction induced by 100 \( \mu \text{M} \) ATP and 30 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) without affecting the contraction induced by 30 mM KCl, suggesting that PLC activation may be involved in \( \text{H}_2\text{O}_2 \)- and ATP-induced responses. Similar result was also observed in rat pulmonary arteries for \( \text{H}_2\text{O}_2 \) [15]. It has also been shown in endothelial cells that U73122, a specific PLC inhibitor, abolished \( \text{H}_2\text{O}_2 \)-induced \( \text{Ca}^{2+} \) mobilization [19], and that \( \text{H}_2\text{O}_2 \) caused hydrolysis of inositol phospholipids [38]. In addition, we have found that neomycin, a chelator of PI(3,4,5)P, [24] or H7, a PKC inhibitor [25], each depressed the peak of the contractions induced by \( \text{H}_2\text{O}_2 \) or ATP. These results, together with the fact that staurosporine, a selective PKC inhibitor, also attenuates \( \text{H}_2\text{O}_2 \)-induced contraction of rat aorta [13], further confirm that ATP- and \( \text{H}_2\text{O}_2 \)-induced contractions share a common P2y-PLC–PKC signaling cascade.

It is well recognized that P2y receptors are coupled also to PLA2 [34,39], and ATP-induced contraction in smooth muscle could be inhibited by indomethacin [37,40]. Our findings that both \( \text{H}_2\text{O}_2 \)- and ATP-induced contractions could be compromised by mepacrine, a PLA2 inhibitor [26], or by indomethacin, a COX inhibitor, again suggest that activation of vascular contraction by \( \text{H}_2\text{O}_2 \) utilizes the same signaling pathway as does ATP.

### 4.4. \( \text{H}_2\text{O}_2 \)- and ATP-induced contractions share common \( \text{Ca}^{2+} \) mobilization pathways

Rat aortic smooth muscles express both P2x and P2y receptors [41]. So, it is now generally believed that ATP triggers intracellular \( \text{Ca}^{2+} \) release via IP3 signaling pathway owned by P2x receptor activation, and that ATP may also elicit extracellular \( \text{Ca}^{2+} \) influx both by direct opening the receptor-operated \( \text{Ca}^{2+} \) channels (ROCC) which is now identified as P2x receptors [42] and by indirect opening the putative store-operated \( \text{Ca}^{2+} \) channels (STOCC) due to P2y receptor activation. On the other hand, it was demonstrated that both extracellular \( \text{Ca}^{2+} \) influx and intracellular \( \text{Ca}^{2+} \) release participate in \( \text{H}_2\text{O}_2 \)-induced \( \text{Ca}^{2+} \) mobilization in rat aortic smooth muscle cells [13]. The present study also showed that both \( \text{H}_2\text{O}_2 \)- and ATP-induced contractions were inhibited by removal of extracellular \( \text{Ca}^{2+} \) or by blockade of intracellular \( \text{Ca}^{2+} \) release with procaine [27] and dantrolene [28]. The fact that Ni\(^{2+} \), an inorganic non-selective \( \text{Ca}^{2+} \) channel blocker, but not nifedipine, a VDCC blocker, also inhibited \( \text{H}_2\text{O}_2 \)- or ATP-induced contractions, suggests the possible involvement of \( \text{Ca}^{2+} \)-influx via the ROCC and/or the STOCC pathway(s) in both contractions. This view is further supported by the fact that both \( \text{H}_2\text{O}_2 \)- and ATP-induced contractions were also inhibited by tetrodramine, a blocker of ROCC and STOCC [29,30]. All these functional results are consistent with the previous notion that \( \text{H}_2\text{O}_2 \) mobilizes cellular \( \text{Ca}^{2+} \) via an agonist-sensitive pathway in endothelial cells, which lack VCDC [18]. At present, however, we cannot reconcile the difference that \( \text{Ca}^{2+} \)-free only partially inhibited and Ni\(^{2+} \) nearly abolished \( \text{H}_2\text{O}_2 \) and ATP-induced contractions. One likely explanation is that Ni\(^{2+} \), besides blocking multiple types \( \text{Ca}^{2+} \) channels, has high affinity for ATP, thus interfering with ATP binding to its receptors. Such an effect of Ni\(^{2+} \) is highly possible, because previous studies had shown that ATP receptors in other cell lines can be inhibited by several multivalent cations, such as La\(^{3+} \) [43], Zn\(^{2+} \) [44] as well as high concentration of Na\(^{+} \) [45].

Oxygen-derived free radicals including \( \text{H}_2\text{O}_2 \) are known to inhibit the SR \( \text{Ca}^{2+} \)-pump in coronary smooth muscle [46–48], which may play a role in the regulation of vascular tone. It is therefore possible that \( \text{H}_2\text{O}_2 \)-induced contraction of rat aorta may be the result of SR \( \text{Ca}^{2+} \)-pump inhibition. However, several lines of evidence suggested that such an effect may play a minor role, if any, in our experimental conditions. First, it was demonstrated that a successful detection of intracellular \( \text{H}_2\text{O}_2 \) in rat aortic smooth muscle cells required the exogenous \( \text{H}_2\text{O}_2 \) at least 100 \( \mu \text{M} \) [49], indicating that if \( \text{H}_2\text{O}_2 \)-induced contraction is truly due to its direct inhibition of SR \( \text{Ca}^{2+} \) pump, then a threshold concentration of 100 \( \mu \text{M} \) must be achieved for \( \text{H}_2\text{O}_2 \)-induced contraction. This is contrast with our observation that as low as 3 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) could elicit a detectable threshold contraction in rat aorta with an EC\(_{50}\) of 30 \( \mu \text{M} \). Furthermore, our observation that a brief pretreatment with 30 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) did not impair PE-induced contraction of rat aorta, would also argue against a significant contribution of SR \( \text{Ca}^{2+} \) pump inhibition on \( \text{H}_2\text{O}_2 \)-induced contraction. Second, in isolated cardiac and skeletal muscle SR vesicles, an earlier study demonstrated that it is the hydroxyl free radical rather than \( \text{H}_2\text{O}_2 \) itself that competitively inhibits the activity of SR \( \text{Ca}^{2+} \) pump [50]. In this context, we had already precluded the involvement of hydroxyl free radical in \( \text{H}_2\text{O}_2 \)-induced contraction. Third, unlike \( \text{H}_2\text{O}_2 \), cyclopiazonic acid (CPA), a selective SR \( \text{Ca}^{2+} \) pump inhibitor in rat aortic smooth muscle [51], triggered a slow and sustained contraction of rat aorta, and this response is not significantly affected by suramin and RB-2 (unpublished observations). Despite all above arguments, we still cannot fully rule out the possibility that the SR \( \text{Ca}^{2+} \)-pump in rat aortic smooth muscles might be somehow impaired if the challenge time and \( \text{H}_2\text{O}_2 \) concentration were increased to 30 min and 250 \( \mu \text{M} \), respectively, as previously employed in the study of coronary smooth muscles [48].

### 5. Conclusions

In summary, we have not only shown that exogenously added \( \text{H}_2\text{O}_2 \) can induce a transient contraction in rat aorta at physiological concentrations, but also for the first time provided pharmacological evidence to suggest that P2x-
purinoceptors activation may be one of the primary signaling steps for H$_2$O$_2$-induced contraction. These findings also bear potential theoretical significance in the study of the activation state of membrane receptors. Indeed, previous studies have shown that several membrane receptors could be directly activated by UV light irradiation [53], and that β-adrenergic receptors can be functionally activated by dithiothreitol, a disulfide-reducing agent [54]. The molecular mechanism(s) of how a ligand-gated ion channel (such as $P_{x}$ receptor) and a G-protein coupled receptor (such as $P_{y}$ receptor) could be activated by an oxidant, such as H$_2$O$_2$, remains to be elucidated.

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References


