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Activation of adenosine A_{2A} but not A_{2B} receptors is involved in uridine adenosine tetraphosphate-induced porcine coronary smooth muscle relaxation

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ABSTRACT

Activation of both adenosine A_{2A} and A_{2B} receptors ($A_{2B}R$) contributes to coronary vasodilation. We previously demonstrated that uridine adenosine tetraphosphate (Up_4A) is a novel vasodilator in the porcine coronary microcirculation, acting mainly on $A_{2A}R$ in smooth muscle cells (SMC). We further investigated whether activation of $A_{2B}R$ is involved in Up_4A-mediated coronary SMC relaxation. Both $A_{2A}R$ and $A_{2B}R$ may stimulate H_2O_2 production leading to activation of K_{ATP} channels in SMCs, we also studied the involvement of H_2O_2 and K_{ATP} channels in Up_4A-mediated effect. Coronary small arteries dissected from the apex of porcine hearts were mounted on wire myograph for Up_4A concentration responses. Up_4A-induced coronary SMC relaxation was attenuated by $A_{2A}R$ but not $A_{2B}R$ antagonism or non-selective P2R antagonism, despite greater endogenous $A_{2B}R$ expression vs. $A_{2A}R$ in both coronary small arteries and primary cultured coronary SMCs. Moreover, Up_4A-induced coronary SMC relaxation was blunted by H_2O_2 catabolism. This effect was not altered by K_{ATP} channel blockade. Combination of H_2O_2 catabolism and $A_{2A}R$ antagonism attenuated Up_4A-induced coronary SMC relaxation to the similar extent as $A_{2A}R$ antagonism alone. Collectively, Up_4A-induced porcine coronary SMC relaxation is mediated by activation of $A_{2A}R$-H_2O_2 pathway. This process does not involve $A_{2B}R$, P2R or K_{ATP} channels.

1. Introduction

Extracellular nucleotides of both mononucleotides and dinucleotides play a pivotal role in the regulation of coronary microcirculation.[1,2] Uridine adenosine tetraphosphate (Up_4A) has been recently identified as the first dinucleotide found in living organisms that contains both purine and pyrimidine parts.[3] Like other extracellular nucleotides, Up_4A exerts its biological effect of regulation of vascular tone in various vascular beds through activation of purinergic receptors.[1,4,5] Purinergic receptors have been classified into two subtypes: P1 receptors (also termed as adenosine receptors) and P2 receptors.[4] In the coronary microcirculation, all four P1 receptor subtypes are expressed in both endothelial and smooth muscle cells (SMC).[6,7] With respect to regulation of vascular tone, activation of A1 and A3 receptors typically results in vascular contraction, whereas activation of A_{2A} and A_{2B} receptors leads to vascular relaxation.[6,7] On the other hand, activation of P2 receptor subtypes e.g. $P2X_1$ receptors on SMCs typically leads to vasoconstriction, while activation of P2 receptor subtypes e.g. $P2Y_1$ receptors on endothelial cells results in vasodilation.[8,9] We previously demonstrated that Up_4A is a potent vasodilator in the porcine coronary microcirculation, which acts mainly through A_{2A} receptors and partially through P2 receptors.[2,10–12] Of interest, a major part of vasodilation produced by Up_4A is mediated by SMC relaxation and activation of SMC $A_{2A}R$.[2] Whether other purinergic P1 receptor subtypes particularly A_{2B} receptors and P2

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receptors are involved in Up_4A-mediated porcine SMC relaxation remain to be determined.

Existing evidence reveals that activation of both adenosine A_{2A} and A_{2B} receptors contributes to coronary vasodilation.[13,14] For post-receptor mechanisms, activation of A_{2A} receptors results in H_2O_2 production leading to an increase in coronary flow.[15] Further, activation of A_{2A}-H_2O_2-K_{ATP} axis accounts for coronary reactive hyperemia.[16] In addition to A_{2A} receptors, activation of A_{2B} receptors regulates coronary flow through K_{ATP} channels.[17] Whether those downstream effectors of purinergic receptors are involved in Up_4A-mediated porcine SMC relaxation deserves further investigation. Consequently, we investigated in the present study whether activation of A_{2B} and P2 receptors are also involved in Up_4A-mediated porcine coronary SMC relaxation using the selective A_{2B} receptor antagonist and the non-selective P2 receptor antagonist. We also addressed the possible involvement of H_2O_2 and K_{ATP} in Up_4A-induced porcine coronary SMC relaxation.

2. Materials and methods

2.1. Drugs and solutions

SCH58261, PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), MRS1754, CVT6883, catalase-polyethylene glycol (Catalase), Glibenclamide, adenosine deaminase (ADA), U46619 (9,11-dideoxy-11α,9α epoxymethanoprostaglandin F2α), and substance P were all purchased from Sigma−Aldrich (Ann Arbor, MI, USA). Up_4A was obtained from Biolog Life Science (Bremen, Germany). SCH58261, MRS1754 and CVT6883 were firstly dissolved in DMSO. All subsequent dilutions (at least 1000 fold) and other drugs were obtained with distilled water. PPADS was protected from light. For the cell culture, Hanks' balanced salt solution, DMEM medium, fetal bovine serum (FBS), antibiotic-antimycotic, collagenase type I, and trypsin inhibitor were purchased from GIBCO (Carlsbad, CA, USA).

2.2. Myograph studies

Porcine hearts ($n = 30$) were collected from a local slaughterhouse (Art's commercial and custom meats, Tunnelton, WV, USA; n = 25, unknown gender, ~100 kg) or from swine used for transplantation course at Karolinska Institutet (n = 5, from female crossbred Yorkshire × Landrace swine, 31−36 Kg). Hearts were kept in Krebs-Henseleit buffer-containing cooler box throughout the transportation. Coronary small arteries (diameter ≈ 150 μm) were dissected out from the apex and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11; pH 7.4. The next day, coronary small arteries were cut into segments of ~2 mm length. In protocols where endothelium-denuded vessels were needed, the endothelium was removed with a single hair by gently rolling it back and forward. Subsequently, vessels were mounted in wire myographs with separated organ baths containing 6 mL Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37 °C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30 min equilibration period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure. Vessels were initially exposed to 30 mM KCl stimulation twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the stable thromboxane A2 analog U46619.[2,18] Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols. In experiments where the effect of an antagonist on the response to Up_4A was assessed, antagonists were added to the organ baths 30 min before preconstriction with U46619 and were present throughout the experiments.[2]

2.3. Experimental protocols

We previously showed that Up_4A mainly activates A_{2A} receptors resulting in coronary relaxation.[2] To investigate the contribution of adenosine - derived from breakdown of Up_4A - to the vasodilator effect of Up_4A, 1 U/mL ADA was applied in the organ bath in which porcine coronary small arteries were exposed to Up_4A concentration responses (10^{-9}-10^{-5} M).[19] To investigate the involvement of purinergic receptors in SMCs, denuded coronary small arteries were preconstricted with U46619 and were subsequently subjected to Up_4A concentration responses in the absence and presence of the A_{2A} receptor antagonist SCH58261 (100 nM),[2] the A_{2B} receptor antagonists MRS1754 (1 μM) and CVT6883 (1 μM),[20] as well as the non-selective P2 receptor antagonist PPADS (10 μM).[2,10] To study the involvement of H_2O_2 and K_{ATP} channels, denuded vessels were exposed to Up_4A in the absence and presence of the H_2O_2 decomposition catalyst catalase (200 U/mL)[21] and the K_{ATP} channel blocker glibenclamide (1 μM).[22] Experiments were also performed in denuded vessels with and without the combination of SCH58261 and catalase.

2.4. Cell cultures

SMCs from porcine coronary small artery were isolated and cultured.[13] Briefly, coronary small arteries from the apex were isolated from five porcine hearts. The endothelial cells were scrapped off by opening up the vasculature and gently rubbing the endothelial surface with metal wire. The denuded vessels were soaked in Hanks' balanced salt solution containing 2% (vol/vol) antibiotic-antimycotic (200 units of penicillin, 200 μg of streptomycin, and 0.5 μg of amphotericin B per mL in final solution) for 15 min. The tissues were then cut into small pieces and digested with enzyme solution containing 1 mg/mL collagenase type I, 0.5 mg/mL soya trypsin inhibitor, 3% bovine serum albumin, and 2% antibiotic. The digested tissues were filtered and collected at 1, 1.5, and 2 h intervals and centrifuged at 1000 rpm for 10 min. The supernatant was discarded. The cell pellets were reconstituted in DMEM with 10% FBS and 2% antibiotic-antimycotic and plated in 100 mm culture plates. The media were replaced once or twice a week during the first week of culturing and every other day in the following weeks. When the cells became confluent, they were split at a 1:5 ratio by using trypsin (0.25%). All the experiments were performed when cells were at the third passage.

2.5. Quantitative real-time PCR analysis

Additional coronary small arteries with intact endothelium and primary cultured porcine arterial SMCs (CASMC) were snap-frozen in liquid nitrogen to be used for detection of A_{2A} and A_{2B} receptor mRNA.[2] Total RNA was extracted using a Qiagen RNA kit. cDNA was synthesized from 100 ng of total RNA with iScript Reverse Transcriptase. Quantitative real-time PCR was performed with SYBR Green. Target gene mRNA levels were expressed relative to the housekeeping gene GAPDH as an endogenous control. The information of primer sequences for A_{2A} receptor, A_{2B} receptor and GAPDH were obtained from previous studies of ours and others.[2,23]

2.6. Data analysis and statistics

Vascular relaxation responses to Up_4A were expressed as percentage of contraction to U46619. The effects of treatment on

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concentration response curve were analyzed with two-way ANOVA (repeated measurement) followed by post hoc analysis using Bonferroni's test. Statistical significance was accepted when $P < 0.05$ (two-tailed). Data are presented as mean ± SEM.

3. Results

3.1. Effects of A_{2B} and P2 receptor blockade on Up_4A-induced porcine coronary SMC relaxation

Up_4A produced potent relaxation in porcine coronary arteries, which was not affected by ADA (Fig. 1A). This indicates a direct vasodilator effect of Up_4A rather than an indirect effect through its degradation to adenosine in coronary small arteries. In accordance with our previous findings,[2] Up_4A-induced coronary relaxation was blunted when the endothelium was removed, yet SMC relaxation still mediated the vasodilator effect produced by Up_4A (Fig. 1B). Interestingly, A_{2A} receptor antagonism with SCH58261 (Fig. 2A) but not A_{2B} receptor antagonism with either MRS1754 (Fig. 2B) or CVT6883 (Fig. 2C) markedly attenuated the Up_4A-induced porcine coronary SMC relaxation, even though the endogenous A_{2B} receptor mRNA expression was much higher than A_{2A} receptor expression in both intact coronary small arteries (Fig. 3A) and primary cultured porcine CASMCs (Fig. 3B). On the other hand, P2 receptor antagonism with the non-selective P2 receptor antagonist PPADS did not affect Up_4A-induced porcine coronary SMC relaxation (Fig. 2D). These findings suggest that activation of A_{2A} but not A_{2B} or P2 receptors contributes to Up_4A-induced porcine coronary SMC relaxation, and Up_4A likely affects A_{2A} receptor sensitivity and/or post-receptor intracellular signaling cascades.

3.2. Effects of H_2O_2 and K_{ATP} blockade on Up_4A-induced porcine coronary SMC relaxation

We previously demonstrated that H_2O_2 plays an important role in A_{2A} receptor-mediated increase in coronary flow[15] and activation of A_{2A}-H_2O_2-K_{ATP} axis accounts for coronary reactive hyperemia in the isolated mouse heart.[16] Consequently, we investigated the involvement of H_2O_2 and K_{ATP} channel in the Up_4A-mediated coronary SMC relaxation in the present study. The H_2O_2 decomposition catalyst catalase partially attenuated Up_4A-induced porcine coronary SMC relaxation (Fig. 4A). This effect was, however, unlikely mediated through K_{ATP} activation, as K_{ATP} inhibitor glibenclamide did not affect Up_4A-induced coronary SMC relaxation (Fig. 4B). Further, the A_{2A} receptor antagonist SCH58261 attenuated Up_4A-induced coronary SMC relaxation to the similar extent as combination of SCH58261 and catalase (Fig. 4C).

4. Discussion

The main findings of the present study focusing on SMCs are that: 1) Up_4A-mediated porcine coronary relaxation was not affected by ADA; 2) Up_4A-induced porcine coronary SMC relaxation was mainly through A_{2A}, but not A_{2B} or P2 receptor activation; 3) the A_{2B} receptor mRNA levels were much greater as compared to A_{2A} receptor expression levels in both coronary small arteries and CASMCs; 4) H_2O_2 catabolism but not K_{ATP} channel blockade affected Up_4A-mediated coronary SMC relaxation; 5) combined H_2O_2 catabolism and A_{2A} antagonism did not further affect Up_4A-induced effect as compared to A_{2A} antagonism alone. The implications of the findings are discussed below.

We previously showed that Up_4A mainly activates A_{2A} receptors resulting in porcine coronary relaxation.[2] The vasodilator effect induced by Up_4A does not appear to be indirect through its degradation to adenosine, as the Up_4A-induced coronary relaxation was not affected by ADA. A major part of coronary vasodilation produced by Up_4A is mediated by SMC relaxation and activation of SMC A_{2A} receptors.[2] In the present study, we further confirmed the significant involvement of SMC A_{2A} receptors in Up_4A-mediated porcine coronary SMC relaxation. In addition to A_{2A} receptors, A_{2B} receptors have also been shown to contribute significantly to relaxation in both mouse and pig coronary vasculature.[13,14] However, the Up_4A-induced porcine coronary SMC relaxation was not affected by two different A_{2B} receptor antagonists, suggesting lack of involvement of A_{2B} receptors. Of interest, the endogenous mRNA expression pattern was similar in intact coronary small arteries and primary cultured porcine CASMCs that the A_{2B} receptor level was much greater as compared to A_{2A} receptors. Although mRNA expression may not accurately reflect protein levels (warranting further investigations), our data suggest that Up_4A likely affects A_{2A} receptor sensitivity and/or triggers the post-receptor intracellular signaling cascade accounting for the Up_4A-mediated coronary SMC relaxation. Altogether, these findings suggest that Up_4A-mediated coronary SMC relaxation is attributed to activation of A_{2A} receptors, and that A_{2B} receptors do not appear to be involved. In addition to P1 receptors, we previously demonstrated that Up_4A-mediated

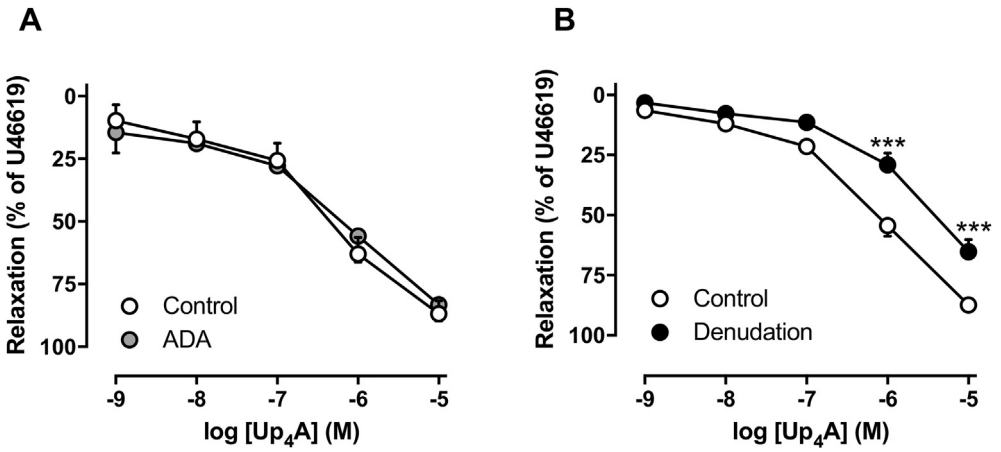

Fig. 1. The effect of adenosine deaminase (ADA) (A, n = 4) and endothelium-denudation (B, n = 17–19) on Up_4A concentration response-induced relaxation in porcine coronary small arteries. Data are mean ± SEM. ***P < 0.001 vs. corresponding control points, calculated with two-way ANOVA followed by post hoc analysis using Bonferroni's test. The experiments were performed in a paired manner in panel A (control vs. ADA).

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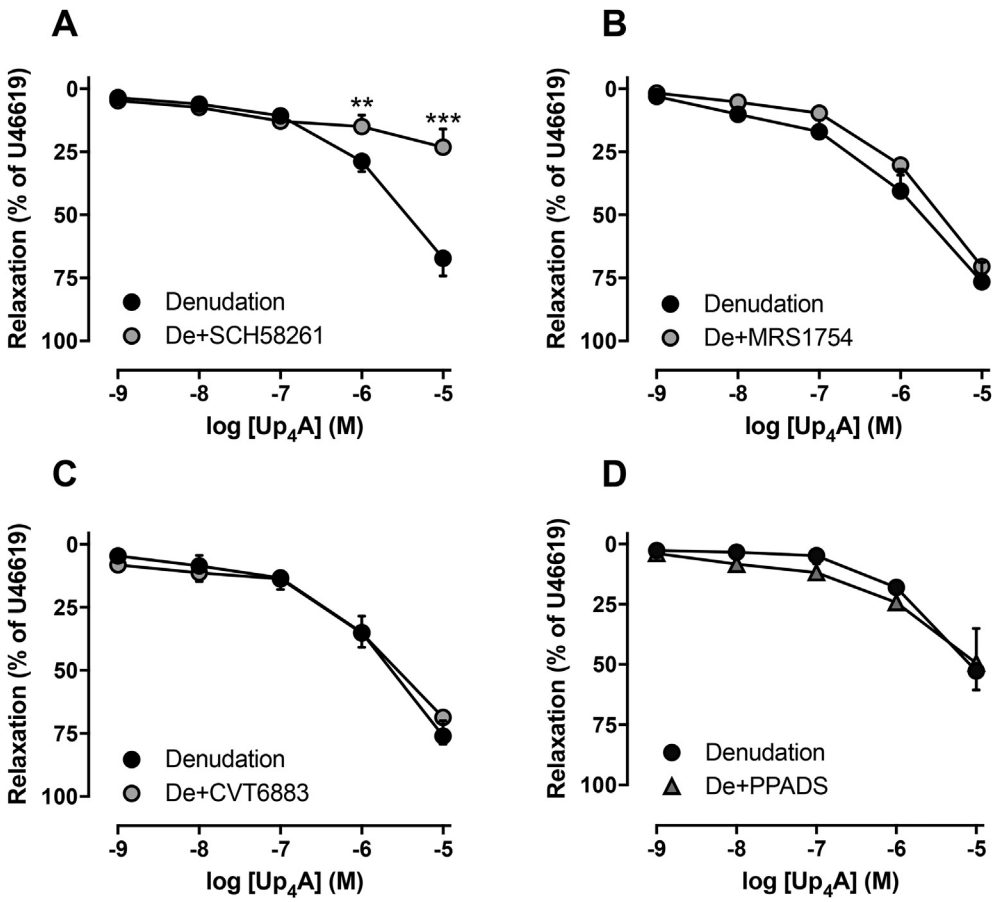

Fig. 2. Up_4A concentration responses in denuded porcine coronary small arteries in the absence and presence of the A_{2A} receptor antagonist SCH58261 (A, n = 9), the A_{2B} receptor antagonist MRS1754 (B, n = 6–8), the A_{2B} receptor antagonist CVT6883 (C, n = 4) and the non-selective P2 receptor antagonist PPADS (D, n = 4–5). Data are mean ± SEM. **P < 0.01, ***P < 0.001 vs. corresponding control points, calculated with two-way ANOVA followed by post hoc analysis using Bonferroni's test. The experiments were performed in a paired manner (denudation vs. inhibitor).

relaxation in porcine coronary small arteries with intact endothelium is partially through activation of P2 receptors.[10–12] However, SMC P2 receptors do not seem to play a role, as the Up_4A-induced coronary SMC relaxation was not affected by the non-selective P2 antagonist.

We previously demonstrated that the $A_{2A}R$-mediated increase in coronary flow requires H_2O_2 production[15] and that activation of A_{2A}-H_2O_2-K_{ATP} axis accounts for coronary reactive hyperemia.[16] In the present study, Up_4A-induced porcine coronary SMC relaxation was partially attenuated by the H_2O_2 decomposition catalyst catalase but was not affected by the K_{ATP} channel blocker glibenclamide. Apparently, the $A_{2A}R$-H_2O_2-K_{ATP} axis is not involved in Up_4A-induced coronary SMC relaxation. In addition to K_{ATP}, H_2O_2 is able to activate BK_{Ca2+} and Kv channels in SMCs leading to coronary vasodilation.[24–27] Future studies, including measurement of SMC membrane potential, are required to determine the involvement of Kv and BK_{Ca2+} channels in Up_4A-mediated coronary SMC relaxation (Fig. 5). As mentioned above, Up_4A may activate post-A_{2A} receptor

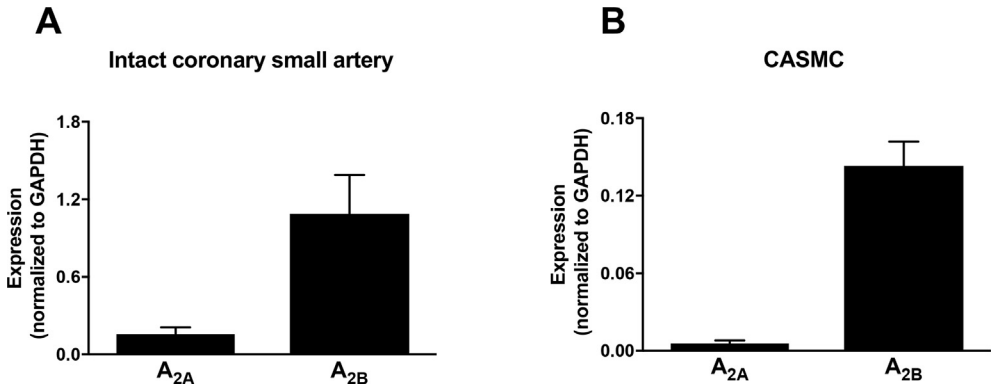

Fig. 3. The mRNA expression for A_{2A} and A_{2B} receptors in intact porcine coronary small arteries (A, n = 6) and primary cultured porcine arterial smooth muscle cells (CASMC) (B, n = 4). Data are mean ± SEM.

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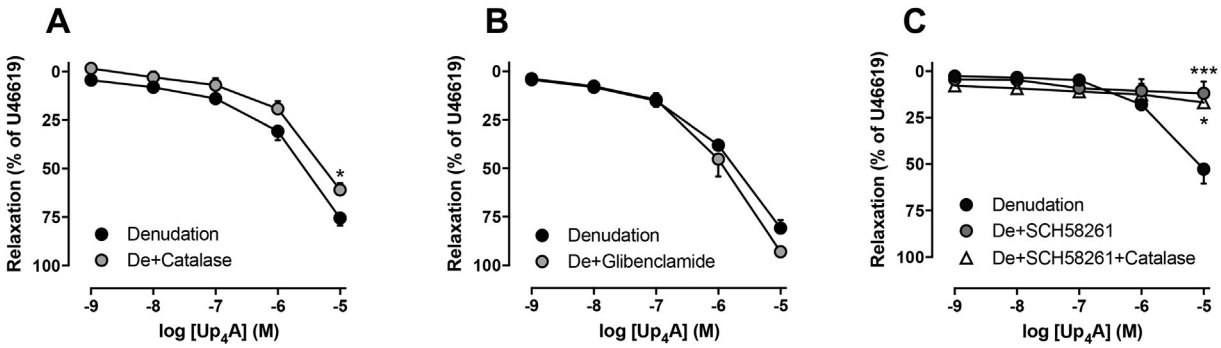

Fig. 4. The effect of the H_2O_2 decomposition catalyst catalase (A, n = 6), the K_{ATP} channel inhibitor glibenclamide (B, n = 5) and combination of the A_{2A} receptor antagonist SCH58261 and catalase (C, n = 4–5) on Up_4A-induced relaxation in denuded porcine coronary small arteries. Data are mean ± SEM. *P < 0.05, ***P < 0.001 vs. corresponding points in denudation group, calculated with two-way ANOVA followed by post hoc analysis using Bonferroni's test. The experiments were performed in a paired manner (denudation vs. inhibitor).

signaling for the SMC relaxation. Indeed, the attenuation in Up_4A-inudced SMC relaxation by A_{2A} receptor antagonism was not affected by addition of catalase, suggesting that activation of A_{2A} receptors may stimulate H_2O_2 accounting in part for the Up_4A-mediated porcine coronary SMC relaxation (Fig. 5). Determination of the exact signaling mechanism for the Up_4A-induced coronary SMC relaxation warrants further investigations.

A limitation in the present study is that coronary small arteries from swine with different age/body weight and genders were used in which the Up_4A-mediated vascular response can be different. We previously demonstrated that Up_4A-induced relaxation in porcine coronary small arteries isolated from slaughterhouse swine (~100 kg, unknown gender) is comparable to those from Yorkshire × Landrace swine (~119 kg, female)[10] or Yorkshire × Landrace swine (~40 kg, either gender).[12] In addition, by comparing Up_4A response in coronary small arteries isolated from swine with known genders, of which the data are included in our previous study,[12] the Up_4A-induced vascular relaxation in coronary vessels was not statistically different between male and female groups (Supplementary Fig. 1). These observations indicate that there is unlikely any age or gender effect on the Up_4A-mediated porcine coronary relaxation.

In conclusion, our findings indicate that Up_4A-induced porcine coronary SMC relaxation is mediated mainly through activation of A_{2A} receptors and partially through H_2O_2. A_{2B}, P2 receptors and K_{ATP} channels do not appear to be activated by Up_4A in porcine coronary SMCs.

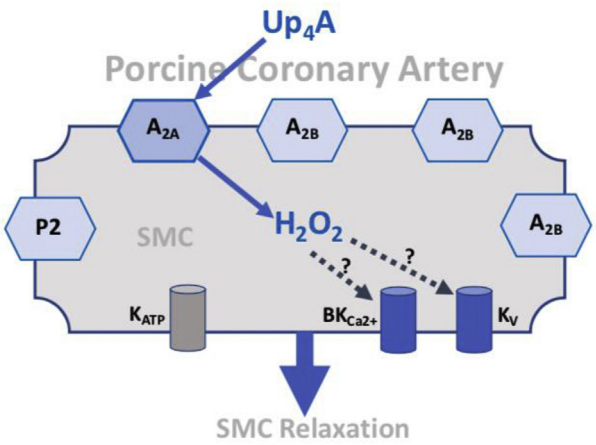

Fig. 5. The schematic illustration summarizes the main findings of the present study that Up_4A mainly activates A_{2A} but not A_{2B} or P2 receptors in porcine coronary smooth muscle cells (SMC) resulting in coronary relaxation. Activation of A_{2A} receptors by Up_4A also stimulates H_2O_2. Activation of K_{ATP} channels is not involved in Up_4A-mediated porcine coronary SMC relaxation.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2019.09.006.

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