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Adenosine A₁ receptors link to smooth muscle contraction via CYP4a, PKC- α , and ERK1/2

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Abstract

Adenosine A₁ receptor (A₁AR) activation contracts smooth muscle, although signaling mechanisms aren't thoroughly understood. Activation of A₁AR leads to metabolism of arachidonic acid, including the production of 20-hydroxyeicosatetraenoic acid (20-HETE) by cytochrome P4504a (CYP4a). 20-HETE can activate protein kinase C- α (PKC- α) which crosstalks with extracellular signal-regulated kinase (ERK1/2) pathway. Both these pathways can regulate smooth muscle contraction, we tested the hypothesis that A₁AR contracts smooth muscle through a pathway involving CYP4a, PKC- α , and ERK1/2. Experiments included isometric tension recordings of aortic contraction and Western blots of signaling molecules in wild type (WT) and A₁AR knockout (A₁KO) mice. Contraction to the A₁-selective agonist CCPA was absent in A₁KO mice aortae, indicating the contractile role of A₁AR. Inhibition of CYP4a (HET0016) abolished CCPA-induced contraction in WT aortae, indicating a critical role for 20-HETE. Both WT and A₁KO mice aortae contracted in response to exogenous 20-HETE. Inhibition of PKC- α (Gö6976) or ERK1/2 (PD98059) attenuated 20-HETE-induced contraction equally, suggesting that ERK1/2 is downstream of PKC- α . Contractions to exogenous 20-HETE were significantly less in A₁KO mice; reduced protein levels of PKC- α , p-ERK1/2, and total ERK1/2 supported this observation. Our data indicate that A₁AR mediates smooth muscle contraction via CYP4a and a PKC- α -ERK1/2 pathway.

Keywords

Cytochrome P4504a; Protein Kinase C- α ; Extracellular Regulated Kinase; 20-HETE

Introduction

Adenosine is an extracellular signaling molecule that affects heart rate, coronary blood flow, and blood pressure (11). Adenosine alters vascular tone by binding to one of the four G-protein coupled receptors: A₁, A_{2A}, A_{2B} and A₃. Activation of A₁ and A₃ receptors contracts vascular smooth muscle, in part, by inhibiting adenylyl cyclase through pertussis toxin-sensitive G_i protein (1, 18, 40). In contrast, activation of A_{2A} and A_{2B} receptors relaxes smooth muscle, in part, by stimulating adenylyl cyclase (1). In the case of A_{2A}, receptors, this process occurs through G_s and G_{olf} proteins, whereas A_{2B} receptors utilize G_s

and G_q proteins (1). In addition to G protein signaling, activation of adenosine receptors also leads to the metabolism of arachidonic acid and the production of myriad signaling molecules (7).

Metabolites of arachidonic acid regulate smooth muscle tone, including prostaglandins, thromboxanes, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) (17, 24, 33). Two subfamilies of cytochrome P450 (CYP) enzymes metabolize arachidonic acid: CYP-epoxygenases and ω -hydroxylases (24). The CYP-epoxygenases produce epoxyeicosatrienoic acids (EETs), which relax smooth muscle. In contrast, the ω -hydroxylases such as CYP4a and CYP4f produce 20 HETE, which contracts smooth muscle (15, 19, 25, 29, 45). The mechanisms linking CYP4a to smooth muscle contraction, however, are not entirely clear, especially in regard to adenosine A₁ receptor activation.

20-HETE-induced contraction of smooth muscle appears to involve protein kinase C (PKC), Src-type tyrosine kinase, rho kinase and mitogen activated protein kinase (MAPK) pathways (19, 27, 38, 43). We have demonstrated previously that A₁ receptor stimulation is linked to PKC- α and ERK1/2 activation in coronary artery smooth muscle (5). Gaps in our knowledge remain to be addressed, the most important of which pertain to whether A₁-dependent activation of PKC- α and ERK1/2 results in smooth muscle contraction. Thus, the aim of the present study was to delineate mechanisms downstream of A₁ receptor activation that lead to smooth muscle contraction. We tested the hypothesis that adenosine A₁ receptors contract smooth muscle through a pathway involving CYP4a, PKC- α , and ERK1/2.

Methods

Animals

Some of the A₁KO mice were obtained from Dr. Jurgen Schnermann (NIDDK, NIH) on C57BL/6 background. C57BL/6 (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Equal number of males and females of 14-18 weeks of age were used in our studies, as no gender differences were observed in the responses to the pharmacological agents used. The Institutional Animal Care and Use Committee of West Virginia University approved this study.

Isometric tension

Following anesthesia with pentobarbital sodium (100 mg/kg, i.p.), the aorta was removed and cut into 3-4 mm rings. Rings were mounted on stainless steel wires and suspended in 10 ml organ baths filled with Krebs-Henseleit buffer containing (in mM): 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose and 2.5 CaCl₂. Baths were maintained at 37°C and bubbled with 95% O₂ and 5% CO₂ (pH 7.4). Rings were equilibrated for 90 min under a resting tension of 1 g. Rings were contracted with 50 mM KCl twice to check viability. Rings were then treated with submaximal phenylephrine (PE; 1 μ M) to obtain a stable contraction and integrity of the vascular endothelium was confirmed by relaxation to acetylcholine (1 μ M). Tension was monitored continuously with a digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC). Our laboratory previously validated all methods (4-5, 28, 40).

Concentration-response curves for 2-chloro-N (6) cyclo-pentyl-adenosine (CCPA; 10 pM to 10 μ M) were run in parallel in aortic rings from WT and A₁KO mice. In all experiments, drugs were administered to yield the next higher concentration only when the previous response reached steady state. 20-HETE (Cayman Chemical; Ann Arbor, MI) concentration-response curves were constructed, but in some experiments a single concentration (100 nM) was used as described previously (2, 27, 36, 44). Inhibitors of CYP4a (HET0016; Cayman

Chemical), PKC- α , (Gö6976; Calbiochem; La Jolla, CA), and ERK1/2 (PD98059; Calbiochem) were added 30 min prior to contraction with PE (10^{-6} M) and present throughout the experiments. Inhibitor concentrations were determined by others and us (3, 5-6, 8, 10, 22-23, 25, 29, 34). Acetylcholine and PE were dissolved in distilled water, while CCPA, HET0016, Gö6976, and PD98059 were dissolved in DMSO. We have shown that previously these vehicles have no effect on smooth muscle contraction (4). 20-HETE was dissolved in ethanol and had no effect on the vasoconstrictive properties of the drug. Percentage of contraction was determined as a percent change from the maximum phenylephrine (PE) response. If A = maximum total tension in the presence of PE; B = maximum total tension when a drug is added after PE; C = minimum passive tension determined after the experiment, after a final wash; D = PE-induced active tension = A minus C. Thus, % contraction = $[(B - A) / D] \times 100\%$

Immunoblots

Aortae from WT and A₁KO mice were homogenized with 150 μ L radio-immuno precipitation assay buffer (RIPA, Cell Signaling Technology; 20 mM Tris-HCl (pH 7.5), (150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin), vortexed, and centrifuged for 10 min at 13,800 g at 4°C. Supernatants were stored at -80°C. Protein was measured using the Bradford dye procedure with bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories; Hercules, CA). The protein extract was divided into aliquots and stored at -80°C. Samples (25 μ g of total protein) were loaded on slab gels (10% acrylamide; 1 mm thick), separated by SDS-PAGE, and transferred to nitrocellulose membranes (Hybond-ECL). Protein transfer was confirmed by visualization of prestained molecular weight markers (Bio-Rad). Membranes were blocked with 5% nonfat dry milk and incubated with primary antibody. 1:1000 dilutions were used for CYP4a (Abcam; Cambridge MA) and PKC- α (BD Transduction Labs; San Diego, CA) antibodies, while 1:500 dilutions were used for total ERK1/2 and p-ERK1/2 (both from Santa Cruz Biotechnology; Santa Cruz, CA). The phospho-ERK1/2 blots were stripped at room temperature for 15 minutes and re-probed for total-ERK1/2. All membranes were stripped and probed for β -actin (Santa Cruz); this served as an internal control to normalize protein expression in each lane. Secondary antibodies were horseradish peroxidase-conjugated. Membranes were developed using enhanced chemiluminescence (AmershamBioSciences) and X-ray film.

Statistical Analysis

Data are expressed as mean $\pm$ SEM from n number of mice. Concentration-response curves and 20-HETE/inhibitor experiments were analyzed by 2-way analysis of variance (ANOVA). Comparisons of two densitometry values were made with unpaired t-tests. $P < 0.05$ was considered significant in all tests.

Results

Role of A₁ receptor and CYP4a product in adenosine-induced smooth muscle contraction

CCPA, an adenosine A₁-selective agonist, contracted aortic rings from WT mice (Fig. 1A, open circles). In contrast, CCPA-induced smooth muscle contraction was absent in aortic rings from A₁KO mice (Fig. 1A, closed circles). ACh-induced response in WT ($47 \pm 2\%$) was not significantly different from A₁KO ($51 \pm 3\%$, $p > 0.05$). HET0016, an inhibitor of CYP4a, attenuated CCPA-induced smooth muscle contraction in aortic rings from WT mice (Fig. 1A, open squares). In contrast, HET0016 had no effect on CCPA responses in aortic rings from A₁KO mice (Fig. 1A, closed squares). There is a significant difference ($p < 0.05$) in PE-induced aortic contraction between WT and A₁KO mice (WT: 0.21 ± 0.01 g; A₁KO:

0.18 ± 0.03 g). However, HET0016 had no significant effect on PE-induced contraction of aortic smooth muscle between WT groups (control: 0.21 ± .01 g vs. treated: 0.2 ± .02 g) and A₁KO mice (control: 0.18 ± .03 g vs. treated: 0.17 ± .02 g). CYP4a protein was expressed in aortae from WT and A₁KO mice (Fig. 1B); however, the expression of CYP4a was lower (by 25%) in A₁KO mice (p<0.05).

Role of 20-HETE in vascular smooth muscle contraction

CYP4a is downstream of adenosine A₁ receptor activation (29), therefore, we determined the contractile responses to its product 20-HETE (0.01-1 μM). Aortic rings from WT and A₁KO mice both contracted in response to exogenous 20-HETE (Fig. 2B and 2C); however, 20-HETE-induced contraction was less in aortae from A₁KO mice (Fig. 2C and 2D). We observed an unexpected increase in the contraction on addition of lowest concentration of 10⁻¹¹M 20-HETE in both WT and A₁KO (Fig. 2B and 2C).

Role of PKC-α in 20-HETE-induced contraction

20-HETE (100 nM), a CYP4a product, contracted aortae from WT and A₁KO mice (Fig. 3A); however, the contractile response was less in aortic rings from A₁KO mice. Inhibition of PKC-α (100 nM Gö6976) reduced 20-HETE-induced contraction in aortic rings from both WT (by 37%) and A₁KO (by 29%) mice (Fig. 3A). The relative inhibitory effect of Gö6976 was the same in aortic rings from WT and A₁KO mice (Fig. 3B). PKC-α protein was expressed in aortae from WT and A₁KO mice (Fig. 3C); however, the expression of PKC-α was lower (by 47%) in A₁KO mice (p<0.05).

Role of ERK1/2 kinase in 20-HETE-induced contraction

The CYP4a product 20-HETE (100 nM) contracted aortic rings from both WT and A₁KO mice (Fig. 3A); however, the contractile response was less in aortae from A₁KO mice. Inhibition of ERK1/2 (1 μM PD98059) reduced 20-HETE-induced contraction in aortae from WT (by 48%) and A₁KO (by 36%) mice (Fig. 3A). The relative inhibitory effect of PD98059 was similar in aortic rings from WT and A₁KO mice (Fig. 3B). ERK1/2 protein was expressed in aortae from WT and A₁KO mice (Fig. 3D); however, the expression of total ERK (by 35%) and phospho-ERK (by 15%) were lower in A₁KO mice (p<0.05).

Discussion

We tested the hypothesis that adenosine A₁ receptors contract smooth muscle through a pathway involving CYP4a, PKC-α, and ERK1/2. We used WT and A₁KO mice, isometric tension recordings of aortic contraction, and Western blots of signaling molecule expression to address this. Our findings regarding an A₁AR → CYP4a → PKC-α → ERK pathway were four-fold. First, CCPA-induced contraction was absent in aortae from A₁KO mice, clearly supporting the idea that adenosine A₁ receptors link to smooth muscle contraction. Second, when CYP4a was inhibited with HET0016, CCPA-induced contraction in WT aortae was abolished. These data uphold the notion that 20-HETE is a critical signaling molecule linking adenosine A₁ receptors to smooth muscle contraction. Further, aortae from both WT and A₁KO mice contracted in response to exogenous 20-HETE. Third, inhibition of PKC-α with Gö6976 attenuated contraction in response to 20-HETE, supporting the idea that PKC-α transduces the signal for contraction. Fourth, inhibition of ERK1/2 with PD98059 attenuated 20-HETE-induced contraction. This provides support for suggesting that ERK1/2 is downstream of PKC-α. Overall, these data indicate that adenosine A₁ receptors mediate smooth muscle contraction via CYP4a (20-HETE) and a PKC-α-ERK1/2 pathway (Fig. 4).

CCPA-induced contraction was absent in A₁KO mice (Fig. 1A); this was not surprising, as others and we have reported it (40, 42). Smooth muscle contractions elicited by A₁ receptors are similar to those found in a wide variety of systems like mouse afferent arterioles (14), human cultured prostatic stromal cells (31), cat esophageal smooth muscle cells (35), guinea pig aorta (12) and mouse coronary artery cells and carotid artery (5, 30, 39). Importantly, however, we observed some unexpected differences between WT and A₁KO mice downstream of the A₁ receptor. We did not have a reason to predict that in A₁KO mice, CYP4a expression would be lower (Fig. 1B) and that responses to exogenous application of the CYP4a metabolite, 20-HETE would be attenuated (Fig. 2). Nor could we have predicted that PKC- α and ERK1/2 (Fig. 3) expression would be reduced in the aortae of A₁KO mice. These findings suggest that genetic ablation of the A₁ receptor leads to some changes in the entire signaling cascade. This is the converse of conditions where A₁ receptors are upregulated and associated with increased expression of PKC and p42/44 ERK (9, 20).

Smooth muscle contraction mediated by A₁ receptors depends almost entirely upon CYP4a products, as HET0016 blocked CCPA-induced contraction (Fig. 1A; Fig. 4). In contrast, HET0016 had no effect on aortae from A₁KO mice (Fig. 1A). These findings suggest that the CYP4a product 20-HETE plays a major role in A₁ receptor signaling. This meshes well with previous studies reported from this laboratory (26), including results obtained by others from rat renal interlobar arteries (37) as well as human and rat cerebral arteries (41). 20-HETE is a potent vasoconstrictor of renal, mesenteric, skeletal and cerebral arterioles in several species (33). In vascular smooth muscle, 20-HETE functions as a second messenger to promote Ca²⁺ influx by depolarization, resulting in contraction (24). Our data show that administration of exogenous 20-HETE contracted aortic rings from both WT and A₁KO mice at low concentrations (Fig. 2B, C and D), with no effect of the solvent. This is concordant with the data shown from other labs as well (13, 15) and underscores that 20-HETE is a potent vasoconstrictor.

The 20-HETE-induced contraction was lower in A₁KO mice, suggesting reduced expression of signaling components downstream of CYP4a. 20-HETE has been shown to signal through PKC in cerebral vascular smooth muscle, renal arterioles and porcine coronary arteries (19, 32, 38). Further, previous studies from our lab have shown that in coronary smooth muscle activation of A₁ receptors is linked to phospholipase C (PLC), PKC- α , and p-ERK1/2 signaling ((5); Fig. 4). We investigated this potential pathway in the aortae of WT and A₁KO mice.

Inhibition of PKC- α (with the isoform-selective Gö6976) attenuated 20-HETE contractions in the aortae of both WT and A₁KO mice (Fig. 3A). Contractions in response to 20-HETE were less in aortic rings from A₁KO mice; however, the relative inhibitory effect of Gö6976 on 20-HETE induced contractions was similar in WT and A₁KO mice (Fig. 3B). This, coupled with reduced expression of PKC- α protein (Fig. 3C), suggests a similar relative role of PKC- α in the contraction of aortae from WT and A₁KO mice. A similar pattern of 20-HETE-induced contractile responses, sensitivity to pharmacological inhibition, and signaling molecule expression was found for ERK1/2 (Fig. 3). Inhibition of ERK1/2 (PD98059) attenuated 20-HETE contractions in the aortae of both WT and A₁KO mice (Fig. 3A). The relative inhibitory effect of PD98059 on 20-HETE induced contractions was similar in WT and A₁KO mice (Fig. 3B). This coupled with reduced expression of phospho-ERK and total ERK (Fig. 3D), suggests similar relative roles of ERK1/2 in the contraction of smooth muscle WT and A₁KO mice. Inhibition of ERK1/2 was more efficacious than inhibition of PKC- α in blocking 20-HETE-induced contraction. This may indicate that the MAPK pathway integrates the input of several relevant signaling pathways including PKC, rho kinases, Ras, and/or tyrosine kinases. There has been no direct evidence showing that PKC α , ERK1/2 and CYP4a are transcriptionally regulated by A₁AR. However, there is

evidence showing that stimulation of adenosine A₁ receptors activates c-fos in Chinese Hamster ovary cells expressing the human A₁ receptor. The transcription factor c-fos regulates the expression of several genes including ERK1/2, PKC- α and PKC- μ (16). Similarly, in human embryonic kidney (HEK-29) cells, stimulation of A₁ receptor coupled to G α induced ERK and STAT1 phosphorylation through a c-fos dependent manner (21). Thus, we can speculate that A₁ receptor may be involved at the transcriptional level as well, but more work is needed to confirm this.

Our current understanding of signaling for A₁ $\rightarrow$ CYP4a $\rightarrow$ PKC- α $\rightarrow$ ERK pathway is summarized in Fig. 4. A₁ receptor activation with, e.g., CCPA, activates CYP4a which metabolizes AA into 20-HETE. CCPA-induced contractions are almost entirely dependent upon signaling through CYP4a, as inhibition with HET0016 abrogates contraction (Fig. 1A). 20-HETE activates PKC- α and this is integral to contraction, as Gö6976 blocks the response (Fig. 3). The ERK1/2 pathway is activated downstream of PKC- α , as Gö6976 blocks ERK activation (5) and PD98059 blocks the contraction (Fig. 3). ERK1/2 integrates the signaling for contraction and transduces it to, e.g., myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), and thin filament regulatory proteins.

In summary, we have shown that A₁ receptor activation leads to smooth muscle contraction via CYP4a, PKC- α , and ERK 1/2. Because A₁ receptors and 20-HETE are implicated in a variety of cardiovascular disorders, a better understanding of this pathway will be important for identifying therapeutic targets and treatment opportunities.

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Fig. 1. Effect of A₁ Adenosine Receptor-induced smooth muscle contraction in WT and A₁ KO aortae

(A) The A₁-selective agonist CCPA-mediated contractions with CYP4a inhibitor (10 μ M HET0016), * p < 0.05 between WT and A₁KO and $^{\dagger}p$ < 0.05 between WT and WT + HET0016 (n=4-12). (B) Western blots for CYP4a expression in the aorta. * p < 0.05 between WT and A₁KO (n=6).

Fig. 2. Effect of the CYP4a product, 20-HETE on aortic smooth muscle contraction from WT and A₁KO

(A) Representative traces for concentration response curve for solvent (ethanol) in WT (n=4). (B) Representative traces for concentration response curve for 20-HETE in WT. (C) Representative traces for concentration response curve for 20-HETE in A₁KO. (D) Exogenous 20-HETE contracts aortic smooth muscle in a concentration-dependent manner. *p < 0.05 between WT and A₁KO mice (n=8-12)

Fig. 3. 20-HETE-induced smooth muscle contraction and its signaling through PKC- α and ERK
 (A) Aortic smooth muscle contractions in response to 100 nM 20-HETE in WT and A₁KO mice with or without PKC- α (100 nM Gö6976) and ERK1/2 kinase (1 μ M PD98059) inhibitors, * p < 0.05 between A₁KO(control/treated) and its respective control; † p < 0.05, WT, A₁KO vs. WT + Gö6976 & A₁KO+ Gö6976 respectively; ‡ p < 0.05, WT, A₁KO vs. WT + PD98059 & A₁KO+ PD98059, respectively, n =8-16. The dagger (†) and double dagger (‡) indicate significant effects of Gö6976 and PD98059 in A₁KO and WT mice, respectively. (B) The inhibitory effect of Gö6976 and PD98059 on 20-HETE-induced contractions are equivalent in WT and A₁KO mice. * p < 0.05 between WT and A₁KO (n =6-8) (C) Western blots for PKC- α expression in the aorta from WT and A₁KO. (D) Western blots for total ERK1/2, phospho-ERK1/2 expression in the aorta from WT and A₁KO mice.* p < 0.05 between WT and A₁KO (n =6-8)

Fig. 4. Adenosine A₁ receptors link to smooth muscle contraction via CYP4a, PKC- α , and ERK1/2

This figure illustrates our understanding of signaling from the A₁ receptor to smooth muscle contraction. Adenosine A₁ receptors (A₁R) are sensitive to CCPA and coupled to phospholipase C (PLC β) by G-proteins (G_X). Candidates for PLC β include PLC β 1, PLC β 3, and PLC γ 1, while candidates for G_X include G_i, G_o, G_q, G₁₁ as well as G- $\beta\gamma$ subunits (5, 18). PLC β produces two second messengers from phosphatidylinositol 4,5 bisphosphate: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃, which releases Ca²⁺ from the sarcoplasmic reticulum; SR). DAG can be metabolized by di- and monoacylglycerol lipases to produce arachidonic acid (AA). CYP4a metabolizes AA into 20-HETE, which activates PKC- α (DAG is also an activator; PKC- β and γ isoforms are also expressed (5). The ERK1/2 pathway is activated downstream of PKC- α . The culmination of this signaling is to contract smooth muscle through effects on, e.g., myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), and thin filament regulatory proteins. Inhibiting CYP4a with HET0016 blocks CCPA-induced smooth muscle contraction (Fig. 1). Gö6976 and PD98059, which inhibit PKC- α and ERK1/2, inhibit smooth muscle contraction in response to 20-HETE (Fig. 3).