CD38 Mediates Angiotensin II–Induced Intracellular Ca^{2+} Release in Rat Pulmonary Arterial Smooth Muscle Cells

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Abstract

CD38 is a multifunctional enzyme that catalyzes the formation of the endogenous Ca^{2+} -mobilizing messengers cyclic ADP-ribose (cADPR) and nicotinic acid adenosine dinucleotide phosphate (NAADP) for the activation of ryanodine receptors (RyRs) of sarcoplasmic reticulum and NAADP-sensitive Ca^{2+} release channels in endolysosomes, respectively. It plays important roles in systemic vascular functions, but there is little information on CD38 in pulmonary arterial smooth muscle cells (PASMCs). Earlier studies suggested a redox-sensing role of CD38 in hypoxic pulmonary vasoconstriction. This study sought to characterize its roles in angiotensin II (Ang II)–induced Ca^{2+} release (AICR) in PASMCs. Examination of CD38 expression in various rat arteries found high levels of CD38 mRNA and protein in pulmonary arteries. The Ang II–elicited Ca^{2+} response consisted of extracellular Ca^{2+} influx and intracellular Ca^{2+} release in PASMCs. AICR activated in the absence of extracellular Ca^{2+} was reduced by pharmacological or siRNA inhibition of CD38, by the cADPR antagonist 8-bromo-cADPR or ryanodine, and by the NAADP antagonist Ned-19 or disruption of endolysosomal Ca^{2+} stores with the vacuolar H^{+} -ATPase inhibitor bafilomycin A1. Suppression of AICR by the inhibitions of cADPR- and NAADP-dependent pathways were nonadditive, indicating interdependence of RyR- and NAADP-gated Ca^{2+} release. Furthermore, AICR was inhibited by the protein kinase C inhibitor staurosporine, the nonspecific NADPH oxidase (NOX)

inhibitors apocynin and diphenyleneiodonium, the NOX2-specific inhibitor gp91ds-tat, and the scavenger of reactive oxygen species (ROS) tempol. These results provide the first evidence that Ang II activates CD38-dependent Ca^{2+} release via the NOX2-ROS pathway in PASMCs.

Keywords: CD38; cyclic ADP-ribose; nicotinic acid adenosine dinucleotide phosphate; angiotensin II; NADPH oxidase

Clinical Relevance

Ca^{2+} signaling in pulmonary arterial smooth muscle cells (PASMCs) is important in many different physiological processes, including contraction, proliferation, migration, and gene transcription. CD38 is a master Ca^{2+} regulator that catalyzes the formation of the endogenous Ca^{2+} -mobilizing messengers cyclic adenosine diphosphate ribose and nicotinic acid adenosine dinucleotide phosphate (NAADP) for the activation of ryanodine receptors of sarcoplasmic reticulum and NAADP-sensitive Ca^{2+} release channels in endolysosomes, respectively. However, its physiological roles in PASMCs have not been examined systematically. This paper provides the first evidence that CD38 is a major player in vasoactive agonist-induced vascular response.

CD38 is a 45-kD transmembrane glycoprotein that is ubiquitously distributed in mammalian tissues, including inflammatory cells, brain tissue, pancreas, cardiac muscles, and airway and vascular

smooth muscle cells (VSMCs) (1). It is a multifunctional enzyme that serves as an ADP-ribosyl cyclase that synthesizes cyclic ADP-ribose (cADPR) from $\beta\text{-NAD}^{+}$ and as a catalase that produces nicotinic acid

adenosine diphosphate (NAADP) from $\beta\text{-NADP}^{+}$ through a base-exchange reaction. cADPR and NAADP can be hydrolyzed also by CD38 to form ADP-ribose and ADP-ribose phosphate, respectively.

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cADPR is the endogenous ligand of ryanodine receptors (RyRs) (2). It binds to the FK506-binding protein, an accessory protein that stabilizes RyRs, causing its dissociation from RyRs to initiate Ca^{2+} release from sarcoplasmic reticulum (SR) (3). NAADP, the most potent Ca^{2+} mobilizing messenger to date, triggers Ca^{2+} release from endolysosomes (4). Recent studies have identified the two-pore channels (TPC1 and TPC2) as the NAADP-activated Ca^{2+} release channels (5, 6). It has been suggested that NAADP binds directly to TPCs or to an accessory protein of the TPC complex to activate endolysosomal Ca^{2+} release (7).

CD38 expression has been reported in some systemic arteries, including aorta and the coronary and renal arteries (8–10). Endogenous vasoconstrictors, such as norepinephrine, endothelin-1 (ET-1), and angiotensin II (Ang II), have been shown to activate the cyclase and catalase activities of CD38, contributing to the elevation of $[\text{Ca}^{2+}]_i$ and vasoconstriction (11–16). In general, these vasoconstrictory agonists of G-protein-coupled receptors activate phospholipase C (PLC) to synthesize inositol trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol biphosphate. IP_3 activates IP_3 receptors to release Ca^{2+} from SR in VSMCs. Evidence from other studies suggests that Ang II and ET-1 can also activate NADPH oxidases (NOXs) to increase the production of reactive oxygen species (ROS) in VSMCs (17). Several studies showed that ROS production is associated with CD38 activation (18–20). These observations suggest that CD38 may play an important role in the regulation of agonist-induced vascular responses.

CD38 expression and its activity have been reported in rat pulmonary arteries (PAs) and pulmonary arterial smooth muscle cells (PASMCs) (21, 22). Previous studies from our and other laboratories have identified the expression of RyR and TPC subtypes and have characterized Ca^{2+} release signals from RyR-gated Ca^{2+} stores (23–25) and NAADP-gated acidic endo-lysosomes in rat PASMCs (13, 21). Hence, all the components of the CD38-dependent Ca^{2+} pathways are operational in PASMCs. CD38 of PASMCs was originally proposed as a redox sensor for hypoxic pulmonary vasoconstriction (HPV) (26). It has been suggested that a reduction of $\beta\text{-NAD}^+:\beta\text{-NADH}$ ratio

during hypoxia stimulates cyclase activity and inhibits hydrolase activity of CD38, leading to the accumulation of cADPR to activate RyR-gated Ca^{2+} release for the sustained HPV (22, 27). This hypothesis has yet to be verified because a recent study failed to observe the contribution of CD38 to HPV in isolated rat PAs without precontracted with a vasoactive agonist (28). In contrast, CD38 may play a significant role in agonist-induced Ca^{2+} release in PASMCs. We have previously shown that the integrin peptide ligand Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) stimulates the production of cADPR and activates Ca^{2+} release from both the

RyR-gated and the endo/lysosomal Ca^{2+} stores (21). Furthermore, ET-1 elicits Ca^{2+} response in PASMCs, partially through NAADP-gated Ca^{2+} release and cross-activation of RyRs by Ca^{2+} -induced Ca^{2+} release (13, 29). Apart from the abovementioned studies, there is no related information on any other vasoactive agonist and on the mechanism underlying agonist-induced activation of CD38 in PASMCs. In this study, we sought to examine the CD38-dependent mechanism in the regulation of pulmonary vascular reactivity by investigating Ang II-induced release (AICR). We compared the expression of CD38 in PAs and systemic

Figure 1. CD38 expression in rat pulmonary and systemic arteries. (A) Verification of the specificity of CD38 antibody in samples of pulmonary artery (PA), renal artery (RA), and cerebral artery (CA) with (right panel) and without (left panel) pretreatment of the antibody with a blocking peptide. (B) An immunoblot of CD38 from samples of aorta, PA, mesenteric (MA), RA, femoral (FA), tail (TA), CA, and pulmonary arterial smooth muscle cells (PASMCs). (C) Averaged normalized CD38 protein level in various arteries using β -actin for normalization ($n = 5$). (D) Quantitative real-time RT-PCR analysis of CD38 mRNA expression in different vascular smooth muscle tissues. The data are normalized with 18S RNA ($n = 5$). a.u., arbitrary units.

arteries, determined the contribution of CD38-dependent Ca^{2+} pathways in AICR, and examined the signaling mechanism of Ang II-induced activation of CD38 in PSMCs. Our results show that Ang II activates CD38-dependent Ca^{2+} release via a protein kinase C (PKC)-NOX2-ROS pathway in PSMCs.

Materials and Methods

PASMC Preparation

All animal procedures are conformed to the Laboratory Animals Care and Use guidelines published by the National Institutes of Health and approved by the Johns Hopkins University Animal Care and Use Committee. PSMCs were isolated from intralobar PAs (300–800 μm) of male Wistar rats (150–200 g) and transiently cultured (18–20 h) as described (13).

Western Blotting

CD38 protein in PAs and PSMCs were determined following a standard protocol (13). Primary antibodies of CD38 (1:500 dilution) (SC-7049; Santa Cruz) and actin (1:5,000 dilution) (SC-1615; Santa Cruz) and horseradish peroxidase-coupled secondary antibody (1:5,000 dilution) (SC-2020; Santa Cruz) were used. A CD38-specific blocking peptide (SC-7049P) was premixed with CD38 antibody (5-fold excess) and incubated overnight at 4°C before use. After CD38 detection, membrane was stripped and reprobed for actin determination. The CD38 value was normalized with actin of each sample to correct for sample variability.

Quantitative RT-PCR

Total RNA from arteries and PSMCs were extracted, and first-strand cDNA was prepared as described (13). Quantitative RT-PCR (qRT-PCR) was performed using primers for 18S rRNA and CD38 of the following sequences: 18S rRNA, forward 5'-CGGCTACCACATCCAAGGAA-3' (X01117.1, position 452–639) and CD38, forward 5'-TGGAGCAAGTCCAAACACCTGGC-3' and reverse 5'-AGCTGGAATTACCGCGGC-3' (NM_013127.1, position 382–500). The qRT-PCR reaction consisted of an initial step at 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute and was

performed using the iQ5 system (Bio-Rad, Hercules, CA). Absolute copy number was calculated using standard curves generated from purified PCR products of known copy number. Data were normalized by the copy number of 18S rRNA in each sample to compensate for sample variability.

Calcium Imaging

PSMCs were loaded with fluo-3 AM, and $[\text{Ca}^{2+}]_i$ was determined using fluorescence microscopy as described (13). $[\text{Ca}^{2+}]_i$ was calculated using the equation: $[\text{Ca}^{2+}]_i = [K_D(F - F_{bg})]/(F_{max} - F)$, where F is the fluorescence intensity, F_{bg} is the background fluorescence, and F_{max} is the maximum fluorescence. The K_D value of fluo-3 is 1.1 μM . F_{max} was determined *in situ* using the Ca^{2+} ionophore 4-Bromo-A23187 (Calbiochem, La Jolla, CA) and 10 mM Ca^{2+} . F_{bg} was measured in an area devoid of cells after Mn^{2+} quenching.

siRNA Knockdown of CD38

Isolated PSMCs were rested in 0.5% FBS-containing HAM's F-12 media

(Mediatech, Herndon, VA) overnight and then cultured in 5% FBS-SmGM (Lonza, Walkersville, MD) for 6 days with two cell passages. Small interfering RNA (siRNA) for CD38 was purchased from Origene (Rockville, MD) (SR509476A, sequence: 5'-ACCAUACCAUGUAACAAGACUCUCU-3') along with the scrambled control sequence. PSMCs were transfected with 100 nM siRNA or scramble control by electroporation using an Amaxa Nucleofactor (Lonza) and immediately seeded on coverslips in 5% FBS-SmGM. After 24 hours, medium was changed to serum-free SmGM for overnight starvation. $[\text{Ca}^{2+}]_i$ measurement and Western blot were performed within 48 hours after the siRNA transfection.

Statistical Analysis

Data are shown as mean $\pm$ SEM. Statistical significance ($P < 0.05$) was assessed by unpaired Student's *t* tests or ANOVA with Holm-Sidak method or Newman-Keuls *post hoc* analyses if applicable.

Figure 2. Nicotinamide (NA) inhibited angiotensin II (Ang II)-induced Ca^{2+} release in rat PSMCs. (A and B) Average Ca^{2+} transient ($\Delta[\text{Ca}^{2+}]_i$) activated by different concentrations of Ang II (10 nM, 100 nM, and 1 μM) in the presence (2 mM Ca^{2+}) or absence (containing 1 mM EGTA) of extracellular Ca^{2+} . (C) The average increase (Δ peak, right panel) in $[\text{Ca}^{2+}]_i$ induced by different concentrations of Ang II ($n = 6$ –7 experiments in each group; $P < 0.05$). *Significant difference between the groups. (D) Averaged traces of Ang II-induced Ca^{2+} transients elicited 100 seconds after removal of Ca^{2+} in the absence or presence of different concentrations (2, 5, 10, and 20 mM) of the CD38 inhibitor NA. (E) Averaged peak change in $[\text{Ca}^{2+}]_i$ in the various groups ($n = 8$ –10 experiments in each group). *Significant differences when compared with control (5 mM, $P = 0.007$; 10 mM, $P < 0.001$; and 20 mM, $P < 0.001$).

Results

Expression Profile of CD38 in Vascular Smooth Muscle

To determine CD38 protein expression in pulmonary and systemic arteries, the specificity of the antibody was first verified using a specific blocking peptide. CD38 was detected as a single band around 45 kD in the resolved protein samples of PA, renal artery (RA), and cerebral artery (CA) (Figure 1A). The signals were completely blocked by the blocking peptide, whereas the nonspecific signals were unaffected. The molecular size of CD38 detected in CA samples was slightly smaller compared with those in PA and RA, presumably due to differences in post-translational modification of glycosylation and phosphorylation. CD38 protein expression in different types of arteries, including aorta, PA, mesenteric artery (MA), femoral artery (FA), tail artery (TA), RA, and CA, as well as isolated PSMCs were examined (Figure 1B). Clear signals of CD38 were detected in PA, RA, CA, and PSMCs compared with the weaker signals in aorta, MA, FA, and TA. Semiquantitative comparison using β -actin for normalization showed the relative abundance of CD38 protein is in the order of CA > RA = PA > MA > aorta = FA = TA (Figure 1C). qRT-PCR showed that the expression profile of CD38 transcript in different types of arteries was similar to that of CD38 protein, although a higher expression level was seen in the aorta (Figure 1D). These results indicate that CD38 protein is differentially expressed in different types of arteries with clear expression in PA and PSMCs.

AICR in PSMCs Was Inhibited by Nicotinamide

CD38 contributes to agonist-induced Ca^{2+} mobilization in several types of cells. Here, we examine the involvement of CD38 in Ang II-induced Ca^{2+} mobilization in PSMCs. Ang II-induced Ca^{2+} response was elicited in the presence extracellular Ca^{2+} or 100 seconds after external solution was switched to Ca^{2+} -free (with 1 mM EGTA) solution (Figures 2A and 2B). Ang II at concentrations of 10 nM to 1 μM elicited a concentration-dependent increase in $[\text{Ca}^{2+}]_i$, which raised rapidly to the peak and returned to the baseline within 50 to 100 seconds. The changes in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$) induced by 10 nM, 100 nM, and 1 μM Ang II were 88.5 ± 32.6 ($n = 6$),

405.6 ± 51.3 ($n = 7$), and 741.4 ± 122.5 nM ($n = 6$), respectively, in the presence of 2 mM Ca^{2+} and 69.6 ± 26 ($n = 6$), 165.5 ± 68 ($n = 7$), and 418.6 ± 126.4 nM ($n = 6$), respectively, in the absence of extracellular Ca^{2+} (Figure 2C). Ca^{2+} responses activated by 100 nM Ang II were significantly reduced after removal of extracellular Ca^{2+} ($P = 0.016$). These results show that Ca^{2+} response elicited by Ang II in PSMCs consisted of Ca^{2+} release and Ca^{2+} influx.

To examine whether Ang II-induced Ca^{2+} release (AICR) is dependent on CD38, PSMCs were incubated for 20 minutes with the CD38 inhibitor nicotinamide (NA) before application of Ang II (100 nM) under Ca^{2+} -free conditions. NA at concentrations between 2 and 20 mM had no significant effect on the basal $[\text{Ca}^{2+}]_i$ in PSMCs but caused concentration-dependent inhibition of AICR (Figures 2D and 2E). $\Delta[\text{Ca}^{2+}]_i$ induced by Ang II was 469.9 ± 60.2 nM ($n = 10$) in the absence of NA and was 375 ± 65.8 ($n = 8$; $P = \text{N.S.}$), 268.9 ± 49.3

Figure 3. Effect of small interfering RNA (siRNA)-mediated suppression of CD38 expression on Ang II-induced Ca^{2+} release (AICR) in PSMCs. (A) An immunoblot of CD38 from samples of PSMCs transfected with a CD38-targeting siRNA and a control scrambled sequence. (B) The mean CD38 protein levels from the two groups after normalization with β -actin ($n = 5$). *Significant decrease in expression compared with the scrambled control ($P = 0.006$). (C) The mean traces of AICR in the control scrambled sequence and CD38 siRNA-transfected PSMCs in the absence or presence of 20 mM NA. (D) The mean peak $\Delta[\text{Ca}^{2+}]_i$ in the control scrambled sequence and CD38 siRNA-transfected PSMCs in the absence or presence of 20 mM NA ($n = 8$ for each group). *Significant difference from the control group in the absence of NA. n.s., no significant difference between the groups indicated.

($n = 8$; $P = 0.007$), 221.7 ± 31.6 ($n = 9$; $P < 0.001$), and 152.9 ± 28.6 nM ($n = 10$; $P < 0.001$) in the presence of 2, 5, 10, and 20 mM NA, respectively. Inhibition of AICR by NA suggests that CD38-dependent Ca^{2+} pathways contribute to the Ang II-mediated Ca^{2+} response in PSMCs.

Knockdown of CD38 Decreased AICR in PSMCs

To further verify the contribution of CD38 to Ang II-induced Ca^{2+} response in PSMCs, AICR was examined in PSMCs transfected with a CD38-specific siRNA or a control sequence. The efficacy of CD38 siRNA treatment was determined by Western blot (Figures 3A and 3B). CD38 protein level was reduced by $69.05 \pm 5.35\%$ in CD38 siRNA-transfected PSMCs compared with that in scrambled sequence transfected cells ($P = 0.006$; $n = 5$). The reduction of CD38 protein expression in the siRNA-transfected PSMCs was associated with attenuated AICR (Figures 3C and 3D). Peak $\Delta[\text{Ca}^{2+}]_i$ values induced

by Ang II (100 nM) were $1,028 \pm 114.5$ nM in control cells ($n = 10$) and 552.6 ± 103.1 nM in CD38 siRNA ($n = 8$) ($P = 0.003$). NA (20 mM) inhibition of AICR, which was clearly observed in the scrambled sequence transfected cells (637.8 ± 119.2 nM [$n = 8$]; $P = 0.012$), was absent in the CD38 siRNA-transfected PSMCs (574.6 ± 71.9 nM [$n = 8$]). Accordingly, the percent reduction in peak $\Delta[\text{Ca}^{2+}]_i$ caused by 20 mM NA was $37.96 \pm 11.59\%$ in the control cells and $-3.97 \pm 13.02\%$ in CD38 siRNA-transfected PSMCs. These results provide the direct evidence that AICR is mediated, in part, through CD38 activation in PSMCs.

AICR in PSMCs Is Mediated in Part by the cADPR-RyR and NAADP-Endolysosomal Pathways

The cyclase activity of CD38 generates cADPR, which causes Ca^{2+} release via RyRs of SR in various types of cells, including VSMCs. To examine the participation of the cADPR-RyRs pathway in PSMCs, we determined the effects of the specific cADPR antagonist 8-bromo-cADPR and ryanodine on the AICR in PSMCs.

Preincubation of PSMCs with various concentrations of 8-bromo-cADPR (0.1–10 μM) for 20 minutes had no effect on the basal $[\text{Ca}^{2+}]_i$ but caused significant reductions in the peak of AICR (Figures 4A and 4B). An apparent maximal inhibition of 40 to 50% of the control response was reached at 1 μM 8-bromo-cADPR. Moreover, inhibition of RyRs with 50 μM ryanodine reduced the peak AICR from 299.5 ± 57.3 nM to 125.4 ± 21.1 nM ($n = 9$; $P = 0.034$) (Figures 4C and 4D). These data suggest that AICR is mediated in part through activation of RyR by cADPR in PSMCs.

In addition to the cADPR-RyRs pathway, CD38 protein also mobilizes Ca^{2+} through production of NAADP, which activates two-pore channels (TPCs) of the endo/lysosomes (5). To evaluate the contribution of the NAADP-lysosomal Ca^{2+} pathways, we examine the AICR in the presence of the specific NAADP antagonist Ned-19. Our previous study showed that 1 μM Ned-19 significantly inhibited intracellular Ca^{2+} release induced by membrane-permeable NAADP-AM in PSMCs without the side effects on RyRs

and IP_3Rs -mediated Ca^{2+} release (13). Preincubation of PSMCs with 1 μM Ned-19 for 20 minutes significantly reduced the peak Ca^{2+} response induced by 100 nM Ang II from 186.9 ± 44 nM to 67.6 ± 14.8 nM ($n = 9$; $P = 0.021$) (Figure 5A). Furthermore, bafilomycin A1 (3 μM), which blocks the vacuolar H^+ -ATPase and disrupts the lysosomal proton gradient for Ca^{2+} uptake (30, 31), significantly suppressed AICR (control: 299.5 ± 57.3 nM [$n = 9$]; bafilomycin A1: 125.4 ± 21.1 nM [$n = 8$]; $P = 0.014$) (Figures 5C and 5D). These results suggest that NAADP-mediated Ca^{2+} release from endo/lysosomal stores also contributes significantly to the Ang II-induced Ca^{2+} response in PSMCs.

CD38-dependent cADPR-RyRs and NAADP-lysosome pathways may operate independently or interdependently to facilitate Ang II-induced Ca^{2+} mobilization. Our previous study provided evidence that Ca^{2+} release from NAADP-sensitive stores is amplified by cross-activation of Ca^{2+} release from RyR-gated Ca^{2+} stores in PSMCs (13). This possibility was examined by inhibiting both pathways with 10 μM 8-Br-cADPR and 1 μM Ned-19. The combined inhibitory effect of 8-Br-cADPR and Ned-19 (peak $\Delta[\text{Ca}^{2+}]_i$: control = 330.4 ± 40.8 nM [$n = 12$]; 8-Br-cADPR+Ned-19 = 157.7 ± 19.5 nM [$n = 10$]; $P = 0.002$) was similar to the inhibition caused by 8-Br-cADPR or Ned-19 alone, indicating that the effects of two inhibitors are nonadditive (Figures 5E and 5F). These results suggest that the two Ca^{2+} release pathways operate interdependently during AICR in PSMCs.

Ang II-Induced CD38 Activation Is Mediated by NOX-Dependent Pathways in PSMCs

To elucidate the signal transduction mechanism of AICR, we examined whether Ang II-induced CD38 activation is mediated by the PKC-NOX-ROS pathway. Preincubation of PSMCs with the PKC inhibitor staurosporine (stau) (10 nM) for 20 minutes significantly reduced the peak AICR induced by 100 nM Ang II from 332.9 ± 30.9 nM ($n = 8$) to 204.7 ± 34.3 nM ($P = 0.019$ [$n = 9$]) (Figures 6A and 6B). Further application of 20 mM NA to inhibit CD38 did not cause additional reduction in the AICR (stau+NA: 163.7 ± 37.2 nM [$n = 10$]; $P = 0.432$). These results

Figure 4. Effects of inhibition of cyclic ADP-ribose (cADPR)-dependent pathway on AICR in PSMCs. (A) The mean traces of AICR in the absence or presence of 1 and 10 μM cADPR antagonist 8-Br-cADPR. (B) The mean peak $\Delta[\text{Ca}^{2+}]_i$ elicited by Ang II in the presence of various concentrations of 8-Br-cADPR ($n = 6$ –9 experiments in each group). (C) Ang II-induced changes in $[\text{Ca}^{2+}]_i$ in the absence or presence of 50 μM ryanodine. (D) The mean peak $\Delta[\text{Ca}^{2+}]_i$ elicited by Ang II with or without preincubation with ryanodine ($n = 9$ experiments). * $P < 0.05$ from control.

Figure 5. Effect of inhibition of nicotinic acid adenosine diphosphate (NAADP)-dependent pathway on AICR in PSMCs. (A) The mean traces of Ang II-induced changes in $[Ca^{2+}]_i$ in the absence or presence of the NAADP antagonist Ned-19 (1 μM). (B) The mean peak changes in $[Ca^{2+}]_i$ in each group ($n = 9$ experiments; $P = 0.021$). (C and D) AICR in PSMCs with or without preincubation with the vacuolar H^+ -ATPase blocker bafilomycin A1 (3 μM) ($n = 8-9$ experiments; $P = 0.014$). (E and F) AICR in PSMCs with or without pretreatment with 8-Br-cADPR (10 μM) and Ned-19 (1 μM) ($n = 10-12$ experiments; $P = 0.002$). *Significantly different from control.

suggest that activation of PKC is required for Ang II-induced CD38 activation.

To determine the contribution of NOX in Ang II-induced CD38 activation, AICR was measured in the absence or presence of the NOX inhibitor apocynin. Peak Ca^{2+} response elicited by Ang II was significantly reduced by 30 μM apocynin (control: 304 ± 56.9 nM [$n = 5$]; apocynin: 96.7 ± 25.1 nM [$n = 6$]; $P < 0.001$). Inhibition of CD38 by NA in the presence of apocynin did not lead to an additional decrease in AICR (apocynin+NA: 111.4 ± 18.1 nM [$n = 6$]) (Figures 6C and 6D). Similarly, inhibition of CD38-dependent Ca^{2+} pathways with 8-Br-cADPR and Ned-19 had no further effect on AICR in the presence apocynin (apocynin: 136.6 ± 29.6 nM [$n = 8$]; apocynin+8-Br-cADPR+Ned-19: 150.1 ± 67.6 nM [$n = 9$]) (Figures 6E and 6F). This suggests that NOX is required for Ang II activation of the cADPR and NAADP pathways. Moreover, AICR was significantly reduced by another NOX inhibitor, diphenyleneiodonium (DPI) (30 μM) (control: 333 ± 30.9 nM [$n = 8$]; DPI: 205.7 ± 34.3 nM [$n = 9$]; $P = 0.019$). NA did not caused an additional decrease

in AICR after inhibition of NOX by DPI (DPI+NA: 164 ± 37.2 nM [$n = 10$]) (Figures 6G and 6H). These results clearly suggest that NOX mediates Ang II-induced CD38 activation in PSMCs.

AICR was examined in the presence of the superoxide scavenger tempol. Preincubation of PSMCs with 100 μM tempol caused significant reduction of AICR (control: 227.6 ± 33.9 nM [$n = 8$]; tempol: 120.5 ± 18.4 nM [$n = 9$]; $P = 0.007$), and addition of NA did not cause further reduction in the Ca^{2+} response (tempol+NA: 92.7 ± 22.4 nM [$n = 9$]) (Figures 6I and 6J). These data indicate that Ang II-induced activation of CD38 is mediated by ROS.

Ang II-Induced Activation of CD38 Is Specifically Mediated by NOX2

Ang II is known to activate NOX1 and NOX2 in other cell types (32, 33). To further examine the specific NOX involvement in Ang II-mediated CD38 activation, we tested AICR in the presence of the NOX1-specific antagonist ML171 and the NOX2-specific inhibitory peptide gp91ds-tat. Exposure to 2 μM ML171 did not suppress

AICR (control: 326 ± 51.3 nM [$n = 6$]; ML171: 315 ± 61.4 nM [$n = 6$]) in PSMCs (Figures 7A and 7B). In contrast, pretreatment of PSMCs with 10 μM gp91ds-tat caused significant reduction of AICR (control: 364.6 ± 38.7 nM [$n = 13$]; gp91ds-tat: 252.4 ± 24.9 nM [$n = 13$]; $P = 0.045$) (Figures 7C and 7D). Furthermore, there was no significant difference between the AICR activated in gp91ds-tat-treated and gp91ds-tat+NA-treated cells (206.5 ± 44.7 nM [$n = 8$]). These results suggest that Ang II-induced CD38-dependent Ca^{2+} release is mainly mediated by the activation of NOX2. Our results suggest that Ang II activates CD38 through PKC-dependent activation of ROS production from NOX2.

Discussion

CD38, through its multifunctional enzymatic activities, generates cADPR and NAADP to mobilize intracellular Ca^{2+} in many different cell types (1). It contributes to agonist-induced vasoconstriction of systemic arteries (16, 34, 35), but its functions in pulmonary vasculature are

unclear. In the present study, we found high expression levels of CD38 protein and mRNA in PA smooth muscle compared with other vascular smooth muscles. Ang II evokes intracellular Ca^{2+} release in part through CD38-dependent cADPR and NAADP pathways in PSMCs. Furthermore, Ang II stimulates CD38 through activation of NOX2. This study hence provides clear evidence that CD38 is a potent regulator of Ca^{2+} homeostasis in pulmonary vasculature and establishes a signaling pathway for AICR in PSMCs.

CD38 expression has been reported in systemic and PAs (8, 21, 22, 35). We found that CD38 protein level varies greatly among different vascular smooth muscles, with the highest level in CA, followed by PA and RA; the expression levels in aorta, MA, FA, and TA are low. This result suggests that the contribution of CD38 to the physiological functions of various vasculatures may be different. Vascular functions of CD38 have not been examined in cerebral arteries despite the abundance of expression, but they are well characterized in renal arteries and arterioles (10, 11, 14, 34, 36). It has been shown that CD38-dependent pathways modulate cytosolic $[\text{Ca}^{2+}]_i$ and contractile functions in renal afferent arteries and arterioles and can account for 50% of the contractile response to Ang II, ET-1, and norepinephrine (10). cADPR-mediated contraction has also been reported in mesenteric arteries (35). The comparable expression of CD38 protein in PA and RA suggests that CD38 may play similarly important role in pulmonary vasculatures.

The contribution of CD38-dependent pathways in Ca^{2+} signaling is clearly evident in PSMCs. Removal of extracellular Ca^{2+} revealed that approximately 50% of the Ang II-induced Ca^{2+} response is mediated by intracellular Ca^{2+} release, suggesting that extracellular Ca^{2+} influx and intracellular Ca^{2+} release are important for Ang II-induced Ca^{2+} mobilization in PSMCs. When focusing on the Ca^{2+} release processes, we found that pharmacological inhibition of CD38 with NA or suppression of CD38 expression with siRNA caused significant inhibition of AICR in PSMCs. NA has been widely used for the inhibition of CD38. The IC_{50} of NA for CD38 inhibition is approximately 1.5 mM in sea urchin (37) and 3.1 mM in rat abdominal aorta (8), as determined by the cyclase assay. NA at 2 to 10 mM also caused

Figure 6. Protein kinase C–NADPH oxidase–reactive oxygen species (PKC–NOX–ROS)-dependent activation of CD38 in PSMCs. (A and B) Mean traces and mean peak $\Delta[\text{Ca}^{2+}]_i$ showing the effect of PKC inhibition with staurosporine (Stau) (10 nM) on AICR in the absence or presence of NA (20 mM) in PSMCs ($n = 8$ –10 experiments in each group). (C and D) Mean traces and mean peak $\Delta[\text{Ca}^{2+}]_i$ showing the effect of the NOX inhibitor apocynin (30 μM) on AICR in the absence or presence of NA ($n = 5$ –6 experiments in each group). (E and F) Mean traces and mean peak $\Delta[\text{Ca}^{2+}]_i$ showing the

Figure 7. Effects of NOX1 and NOX2 specific antagonist on AICR in PSMCs. (A and B) The effect of the NOX1-specific inhibitor ML171 (30 μ M) on AICR in PSMCs. (C and D) The effects of the NOX2-specific peptide inhibitor gp91 ds-tat (10 μ M) on AICR in PSMCs in the absence or presence of NA ($n = 8$ –13 experiments in each group). (E) A schematic diagram showing the proposed signaling pathways for AICR. *Significant inhibition of AICR compared with control. There was no significant difference between gp91 ds-tat and gp91 ds-tat+NA-treated cells. CICR, Ca^{2+} -induced Ca^{2+} release; NAADP, nicotinic acid adenosine dinucleotide phosphate; RyR, ryanodine receptor; TPC, two-pore channel.

concentration-dependent inhibition of CD38 cyclase activity in homogenate of coronary artery (38) and effectively inhibited agonist-induced vasoconstriction in systemic arteries and the Ca^{2+} response in arterial myocytes (16, 34, 35). The concentration-dependent inhibition of AICR by NA in PSMCs is therefore consistent with CD38 inhibition reported in systemic vascular smooth muscle. Moreover, siRNA knockdown of CD38 caused similar inhibition of AICR compared with NA, and NA failed to cause further reduction of AICR in CD38 siRNA-transfected cells. These results suggest that CD38 plays a significant role in the agonist-induced Ca^{2+} release in PSMCs and that NA does not cause major side effect on Ca^{2+} release independent of CD38 under our experimental conditions.

CD38 activation mobilizes intracellular Ca^{2+} through cADPR-dependent Ca^{2+}

release from RyR-gated stores and NAADP-dependent Ca^{2+} release from the acidic endo-lysosomes. Previous work by our and other laboratories have identified all three RyR subtypes in PSMCs, with RyR2 being the most abundant subtype (23, 39). RyR1 and RyR2 are associated mainly with SR in the subsarcolemmal region, whereas RyR3 is localized predominantly in the perinuclear SR (23, 40). Mild activation of RyRs triggers discernible local Ca^{2+} events or Ca^{2+} sparks, and strong activation can lead to regenerative global Ca^{2+} release in PSMCs (23–25). NAADP-mediated Ca^{2+} release has also been described in PSMCs (5). We have recently identified the expression of NAADP-sensitive TPC1 and TPC2 in PSMCs and have characterized the NAADP-mediated global and local Ca^{2+} events using the membrane-permeable NAADP-AM (13, 40). The NAADP-mediated Ca^{2+} release

in PSMCs is initiated from bafilomycin-sensitive acidic endo-lysosomes and is blocked by the specific NAADP antagonist Ned-19, which does not affect the voltage-dependent Ca^{2+} entry, store-operated Ca^{2+} entry, and RyR-gated and IP_3 -gated Ca^{2+} release in PSMCs (13, 41). These studies hence show that all the effectors of the CD38-dependent cADPR and NAADP pathways are operational in PSMCs.

In this study, antagonism of cADPR with the 8-bromo-cADPR or inhibition of RyRs with ryanodine effectively suppressed AICR. Inhibition of NAADP with Ned-19 (41) or depleting endo/lysosomal Ca^{2+} stores by disrupting H^+ gradient for H^+ - Ca^{2+} exchange with the vacuolar H^+ -ATPase inhibitor bafilomycin A1 also reduced AICR. These results suggest that Ang II stimulates both the cyclase and catalase activities of CD38 to generate cADPR and NAADP, respectively, for

Figure 6. (Continued). effect of apocynin (30 μ M) on AICR in the absence or presence of 8-Br-cADPR (10 μ M) and Ned-19 (1 μ M) ($n = 8$ –9 experiments in each group). (G and H) The effect of the NOX inhibitor diphenyleneiodonium (DPI) (30 μ M) on AICR in the absence or presence of NA ($n = 8$ –10 experiments in each group). (I and J) The effect of the ROS scavenger tempol (100 μ M) on AICR in the absence or presence of NA ($n = 9$ experiments in each group). *Significant inhibition of AICR compared with control. There was no additional significant inhibition of AICR in cells treated with NA compared with staurosporine, apocynin, DPI, or tempol alone.

eliciting the Ca^{2+} response. The cADPR- and NAADP-dependent mechanisms operate interdependently during AICR. This is reflected in the similar suppression of AICR by coinhibition of cADPR and NAADP pathways, compared with the inhibition of either the cADPR or the NAADP pathway alone. The interaction between the two Ca^{2+} pathways is consistent with others and our previous findings of cross-activation of RyRs by NAADP-mediated Ca^{2+} release in PSMCs (13, 29, 42). NAADP channels are spatially or functional coupled with RyRs through Ca^{2+} -induced Ca^{2+} release (13, 29, 40), and cADPR may further sensitize RyRs for the cross-activation under physiological stimulation. The remaining AICR in PSMCs after coinhibition of cADPR and NAADP is likely mediated by IP_3 -gated Ca^{2+} release through the well-recognized PLC- IP_3 pathway.

There is substantial evidence suggesting that Ang II enhances ROS production through activation of NOX, contributing to Ca^{2+} response in VSMCs (17). Several studies have shown that CD38 activity is regulated by redox state and oxidative stress (19, 20, 22, 27, 43). However, the mechanism of Ang II-induced CD38 activation in PSMCs has not been established. We found that Ang II activates CD38-dependent Ca^{2+} release in PSMCs via ROS generation by NOX. This is based on the findings that AICR was inhibited significantly by the common NOX inhibitors apocynin and DPI and by the ROS scavenger tempol. Inhibition of CD38 with NA, or inhibition of the cADPR and NAADP pathways, in the presence of the

NOX inhibitors or ROS scavenger did not further suppress AICR. These observations are consistent with reports in renal afferent arterioles and cardiac myocytes that Ang II stimulates NOX to generate ROS, leading to enhanced ADP-ribosyl cyclase activity (36, 44).

There are seven NOX homologs (NOX1–5 and DUOX1–2) with different subunit compositions, physiological functions, and activation mechanisms (45). Multiple isoforms of NOX, including NOX1, NOX2, and NOX4, are expressed in systemic arteries (17, 45) and PAs (46, 47). Ang II-induced activation of NOX1 appears to be important in systemic VSMCs of large arteries, whereas NOX2 may be more important in resistance arteries (32, 33). Ang II activates NOX2 by PKC-dependent phosphorylation of the cytosolic subunit p47phox to facilitate the assembly of the active oxidase complex (17). NOX1 and NOX2 activation is also critically modulated by the small GTP-binding protein Rac1 (17, 48, 49). It has been shown in coronary arteries that ET-1 stimulates Rac1-NOX1-dependent ROS production to activate CD38 (49). In this study, preincubation of PSMCs with the specific NOX1 inhibitor ML171 had no significant inhibitory effect on AICR. In contrast, the NOX2-specific peptide inhibitor gp91ds-tat caused a significant decrease in AICR. Furthermore, inhibition of CD38 by NA in the presence of gp91 ds-tat did not cause further reduction in the Ang II-induced response. This suggests that Ang II-induced activation of CD38 in PSMCs is mediated specifically by NOX2. Moreover, suppression of AICR with the

PKC inhibitor staurosporine is consistent with Ang II-induced CD38 activation through the PKC-NOX2-ROS pathway to elicit Ca^{2+} release in PSMCs. However, Ang II activation of NOX2 may require other signaling pathways, such as Rac1 (17, 48), and PKC may also be modulated by the CD38 signaling pathways downstream of NOX2. CD38 of PSMCs can be activated through mechanisms unrelated to the PKC-NOX-ROS pathway. For example, activation of integrins with the GRGDSP peptide can elicit a CD38-dependent Ca^{2+} response in PSMCs (21). Because integrins are not G-protein-coupled receptors and because ligation of integrins activates complex signaling pathways, including focal adhesion kinase, integrin-linked kinase, receptor tyrosine kinase, and Src (50), it is likely that integrins activate CD38 through mechanisms different from Ang II.

In conclusion, we have characterized systematically the mechanism of Ca^{2+} release induced by Ang II and have demonstrated that it is in part mediated by CD38 activation through NOX2-dependent ROS production, leading to synergistic Ca^{2+} release from cADPR- and NAADP-gated Ca^{2+} stores of PSMCs. A schematic diagram illustrating the proposed mechanism for Ang II-induced activation of CD38-dependent Ca^{2+} release is shown in Figure 7E. These findings provide the evidence to support an important role of CD38 for Ca^{2+} signaling in pulmonary vasculature. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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