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H_2O_2 activates Nox4 through PLA_2-dependent arachidonic acid production in adult cardiac fibroblasts

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Abstract Stimulated production of reactive oxygen species (ROS) by plasma membrane-associated nicotinamide adenine dinucleotide phosphate oxidases (Nox) in non-phagocytic cells regulates a number of biological processes including growth, vessel tone, and oxygen sensing. The purpose of this study was to investigate H_2O_2-stimulated ROS production in primary adult cardiac fibroblasts (CF). Results demonstrate that CF express an H_2O_2-inducible oxidant generating system that is inhibitable by diphenylene iodonium (DPI) and sensitive to antioxidants. In addition to H_2O_2, generation of ROS was stimulated potently by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and arachidonic acid (AA) in a protein kinase C-independent manner. Pretreatment with arachidonyl trifluoromethyl ketone was nearly as effective as DPI at reducing H_2O_2- and OAG-stimulated oxidant generation indicating a central role for phospholipase A_2 (PLA_2) in this signaling pathway. Co-stimulation with H_2O_2 and OAG did not increase ROS generation as compared to OAG alone suggesting both agonists signal through a shared, rate-limited enzymatic pathway involving PLA_2. Co-stimulation with H_2O_2 and AA had additive effects indicating these two agonists stimulate oxidant production through a parallel activation pathway. Reverse transcriptase-coupled polymerase chain reaction and Western blotting demonstrate primary cardiac fibroblasts express transcripts and protein for Nox4, p22, p47, and p67 phox. Transfections with Nox4 small inhibitory ribonucleic acid oligonucleotides or p22 phox antisense oligonucleotides significantly downregulated stimulated Nox activity. Inhibitors of nitric oxide synthases were without effect. We conclude adult CF express Nox4/p22 phox-containing oxidant generating complex activated by H_2O_2, OAG, and AA through a pathway that requires activation of PLA_2.

Keywords: Nox4; NADPH oxidase; Reactive oxygen species; Cardiac fibroblasts; Arachidonic acid

1. Introduction

Traditional thinking views fibroblasts as primarily structural elements associated with modulation of extracellular matrix and tissue healing/repair. We [1] and others [2,3] have shown primary cardiac fibroblasts (CF) produce cytokines, chemokines and other inflammatory markers, and data suggest they serve as oxygen sensors in some tissues [4]. Moreover, emerging data suggest tissue specific differences in CF properties, in particular their responses to stress. In the heart, elevated reactive oxygen species (ROS) are transiently generated in ischemic tissue upon reperfusion (I/R) [5,6]. We recently showed exposure of primary adult cardiac fibroblasts (CF) to a brief pulse of physiological levels of H_2O_2 results in large delayed Ca^{2+} fluxes and Ca^{2+}-dependent interleukin-6 release [1]. In addition, we observed transient p38 MAP kinase and ERK1/2 activations, as well as phenotypic changes marked by reduced migration and decreased apoptotic cell death [7]. These results demonstrate myocardial I/R stimulated ROS generation alters CF phenotype and initiates an inflammatory response. The source of the ROS burst and the mechanisms of ROS-initiated intracellular signaling in CF are not well understood.

Non-phagocytic cells express membrane-associated NAD(P)H oxidase (Nox) enzymes analogous to the gp91/Nox2-containing phagocytic plasma membrane-associated NADPH oxidase [8]. The functions of these enzymes are diverse and differ significantly from the phagocytic enzyme with respect to mechanism of activation and amount of O_2^- produced. While phagocytes produce large quantities of O_2^- necessary for their crucial role in killing pathogens, many non-phagocytic cells generate lower levels of O_2^- in response to various stimuli including H_2O_2, which likely function as intracellular and paracrine signaling molecules [9]. For example, angiotensin II (AII) has been shown to induce TGF-β expression in ventricular myocytes in an ROS-dependent manner through the sequential activations of NADPH oxidase, PKC, p38 MAP kinase and ultimately the redox-sensitive transcription factor AP-1 [10]. Microvascular endothelial cells generate NADPH oxidase-dependent ROS in response to TNF-α and phorbol esters [11]. These studies and others [12–14] demonstrate the central importance of ROS in mediating intracellular signaling.

The purpose of this study was to investigate ROS-induced ROS production in primary adult CF. Furthermore, we sought

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Abbreviations: CF, cardiac fibroblasts; Nox, NAD(P)H oxidase; ROS, reactive oxygen species; DPI, diphenylene iodonium; AA, Arachidonic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; AATFMK, arachidonyl trifluoromethyl ketone; PLA_2, phospholipase A_2; phox, phagocyte oxidase; DCF, 2′,7′-dichlorofluorescein diacetate; BIM, bisindolemaleimide; STA, staurosporine

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to identify the primary source(s) of stimulated ROS and characterize activation mechanisms of the oxidase. Our results demonstrate that CF express an ROS generating system that is activated by H_2O_2, diacylglycerols, and arachidonic acid (AA) through a mechanism that requires activation of $cPLA_2$, is protein kinase C independent, and is highly sensitive to diphenylene iodonium (DPI) and antioxidants. We show for the first time that CF express mRNA and protein for Nox4 and its putative subunits suggesting a role for this enzyme in stimulated ROS generation and oxidant signaling by CF.

2. Materials and methods

2.1. Fibroblast isolation and culture

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health [15]. Cardiac-derived fibroblasts were isolated from the hearts of adult male WKY rats (200–250 g) using a method developed in our laboratory [1,7]. Briefly, after induction of deep anesthesia with an i.m. injection (0.2–0.3 ml) containing a cocktail of ketamine–acepromazine–xylazine (9:3:1), hearts were rapidly removed, rinsed, and mounted via the aorta onto an 18-guage cannula attached to a Langendorff-type apparatus allowing retrograde perfusion of the coronary arteries. Hearts were perfused for 5 min with 37 °C sterile-filtered calcium-free Krebs–Ringers–bicarbonate buffer (KRB; NaCl 110 mmol/L, KCl 2.6 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, and glucose 11 mmol/L) at 80 mmHg. Hearts were perfused for a further 20–25 min with KRB-enzyme solution containing 0.5 mg/ml type II collagenase (Worthington Biochemical, Corp., Freehold, NJ), 25 μmol/L $CaCl_2$, and 1 mg/ml fatty-acid free albumin. After digestion, the ventricles were trimmed free and minced in KRB-enzyme solution containing 10 mg/ml albumin, filtered through sterile nylon mesh and centrifuged at $25 \times g$ for 90 s to remove cardiomyocytes, RBCs and debris. The resultant supernatant was then centrifuged at $1000 \times g$ for 8 min. The cell pellet was resuspended in 20 ml CF medium (CF; HEPES 15 mmol/L, $NaHCO_3$ 16.7 mmol/L, BME-vitamins and MEM-amino acids each 1× [GIBCO BRL, Grand Island, NY], glutamine 2 mM, heat-inactivated FBS 10%, and antibiotics, pH 7.3) and plated into T175 tissue-culture flasks (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ). Non-adherent cells were removed by aspiration after 2 h and discarded. Cells were fed with fresh medium three times per week using CF medium and split 1:2 when confluent.

CF and non-fibroblast contaminants were identified by immunofluorescence (IF) using routine methods [16] with the following antibodies; FITC-conjugated monoclonal anti-β-actin (Sigma Chemical Co., St. Louis, MO), anti-PECAm-1 (CD-31; Research Diagnostics, Flanders, NJ), anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-smooth muscle actin (Sigma). After 2 serial passages >99.7% of cells in these cultures exhibited vimentin and β-actin immunoreactivity, were CD-31 and smooth muscle actin negative, and displayed typical fibroblast-like morphology. Non-fibroblast cells typically account for less than 0.1% of total cells as determined by IF. Cardiac-derived fibroblasts were used in these experiments between the second and third passages.

2.2. Intra- and extracellular ROS measurements

Intracellular ROS was monitored using the ROS-sensitive fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCF; [17]). CF were plated into clear bottom, black walled 96-well plates (Corning Inc., Corning N.Y.) at 1500 cells/well. CF were loaded for 20 min with 10 μM DCF in buffer (137 mM NaCl, 1.2 mM $MgSO_4$, 4.9 mM KCl, 1.2 mM NaH_2PO_4, 20 mM HEPES, 15 mM glucose, 1.8 mM $CaCl_2$, pH 7.4), washed and read in a microplate fluorometer/luminometer with 485/20 excitation and 528/20 emission filters (FL_x800i, Bio-Tek Instruments, Winooski, VT). DCF fluorescence was monitored for 1 h, stored on microcomputer and analyzed using KC^4 software (Bio-Tek). The rate of ROS production was defined as the slope of the linear portion of the fluorescence curve for each test well (typically linear >20 min). The plates were then frozen at −80 °C for 2 h, thawed and stained with the nucleic acid-sensitive CyQuant GR dye according to the manufacturer's protocol (Molecular Probes, Eugene, OR). This allowed normalization of ROS generation rates to DNA content. Data are expressed as the ratio of the rate of DCF fluorescence to CyQuant fluorescence.

Extracellular ROS levels were monitored using an enhanced luminol-based assay (LumiMax, Stratagene, La Jolla, CA). CF were plated into 96-well plates as described above, stimulated and assayed for superoxide anion using the manufacturer's protocol.

2.3. Western blotting

Extraction of protein homogenates, Western blotting, and autoradiography were performed as previously described [1,18]. The following antibodies and dilutions were used: rabbit anti-Nox4 (1:1000; kind gift from Dr. Hanna Abboud), rabbit anti-p22 phagocyte oxidase (phox) (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA; FL-195), goat anti-p47 phox (1:2500; Santa Cruz; C-20), rabbit anti-p67 phox (1:2500; Santa Cruz; H-300), goat anti-gp91/Nox2 (1:2000; Santa Cruz; C-15). Briefly, 10-20 μg total protein/lane was separated on 12% SDS–PAGE gels, blotted onto nitrocellulose and detected by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ). Molecular weights of phox subunits were estimated from prestained high and low molecular weight markers (BioRad).

2.4. RT-PCR detection of phox subunits

Total RNA was isolated from CF grown in 100 mm culture dishes using TriZol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (0.5–1 μg) was reverse transcribed and amplified using the Titan One Tube RT-PCR system (Roche Applied Science, Indianapolis, IN) using the oligonucleotide primers shown in Table 1. Following reverse transcription and PCR the amplicons were separated on 2% agarose gels, stained with ethidium bromide and photographed.

2.5. p22 phox Antisense and Nox4 siRNA transfections

CF grown to 50% confluency in 6-well plates were transfected with the following p22 phox oligonucleotides (200 nM) using oligofectamine reagent (Invitrogen): sense 5′-GGTCCTCACCATGGGGCAGATC-3′, antisense 5′-GATCTGCCCCATGGTGAGGACC-3′,

Table 1
RT-PCR primer sequences

Transcript	Sense (5′–3′)	Antisense (5′–3′)	Accession No.
Nox1	TGTGTGTCGAAATCTGCTCTCC	TGAGAACCAAAGCCACAGTG	NM_053683
Nox2	TATTGTGGGAGACTGGACTG	GATTGGCCTGAGATTCATCC	AY174116
Nox3	GTATTTGCTGCAGAGGAC	ATTCTGCATAGCGGTCTC	AF190122
Nox4	GTTCCAAGCTCATTTCCCAC	GTATCGATGCAAACGGAGTG	NM_053524
Nox5	GGGCAAGAATGACATGAAGG	TCTGTTGAGCAGGATTAGGG	AF317889
p22phox	CATTGCCAGTGTGATCTACC	ATTACAGTGGGCATCACC	AJ295951
p47phox	CCACACCTCTTGAACTTC	GCCATCTAGGAGCTTATG	AF260779
p67phox	CCACTCGAGGATTTGCTTCA	ATCTTGGAATGCCTGGGCTC	XM_344156
p40phox	GCTGACATCGAGGAGAAGA	AGGATCTTCACGAAGGACC	AB002665
Noxo1	GCCTGCTACGGAGATCTGA	TTCTCCACCAGCCACCAGCCT	AF539797
Noxa1	GCTGCGGGCATTTGACCAA	AAGGAAATCCATGGGCTCCA	AY25570

scrambled 5′-TGGCGAGGTGCCCAGTTCAACC-3′. In separate experiments, FITC-labeled oligonucleotides were transfected into CF to verify and monitor uptake. CF were transfected with the following two Nox4 siRNAs (Ambion) at 100 nM final concentration each: siRNA1 5′-GUUAGUCUGUGUGUGGCUGtt-3′, siRNA2 5′-GAUUUGCCUGGAAGAACCCtt-3′ as described [19].

2.6. Arachidonic acid release

Primary CF were grown to 90% confluency in 6-well plates (Corning). CF were labeled overnight with [^{3}H]arachidonic acid (0.5 μCi/ml; DuPont NEN Research Products, Boston, MA) in CF medium containing 10% serum. Cells were washed three times and stimulated with H_2O_2 (100 μM, 3 min), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 100 μM), or buffer (control). After 1 h medium was collected and analyzed for [3H]AA content by scintillation counting. Raw CPM were converted to DPM and normalized to DNA content (CyQuant Assay).

2.7. Statistical analysis

Values represent means ± S.D. Statistical significance was assessed by ANOVA with Bonferroni-corrected Student's *t* test. P values $\leqslant 0.05$ were considered significant.

3. Results

Exposure of CF to a 3 min pulse of H_2O_2 stimulated endogenous ROS production. As shown in Fig. 1A, brief exposure of CF to >10 μM H_2O_2 led to dose-dependent increases in the rate of ROS generation whereas lower doses were without effect. Hydrogen peroxide readily diffuses across cell membranes and may directly stimulate DCF fluorescence especially during sustained exposures. However, given the brevity of the stimulus (3 min), the inclusion of catalase (500 U/ml) in the test buffer in selected experiments to scavenge any residual exogenous H_2O_2, and the duration of DCF fluorescence monitoring (1 h), the effects of applied H_2O_2 on the kinetics of ROS generation is likely the result of stimulated endogenous production rather than any direct oxidation of the fluorescent probe. Pretreatment with DPI, a flavin-containing enzyme inhibitor, effectively blocked peroxide-induced ROS production implicating an NADPH oxidase (Nox, Duox) and/or nitric oxide synthase (NOS) as possible sources of ROS. Since DCF is unable to discriminate between several intracellular ROS species including H_2O_2 and NO [17], we sought to define the relative contribution of NO to the ROS signal. Pretreatment of CF with various NOS inhibitors including L-NAME (100 μM) and 1400 W (5 μM) had no effect on peroxide-induced DCF fluorescence. Furthermore, metabolites of NO were undetectable in culture supernatants after 48 h using colormetric and fluorometric assays (not shown). These data suggest that NO does not contribute substantially to the observed ROS signal. Similarly, extracellular ROS levels remained below threshold detection limits using a luminol-based assay.

Activation of protein kinase C (PKC) is known to stimulate ROS production via Nox in many cell types. However, treatment with various concentrations of phorbol ester (PMA) failed to stimulate ROS generation. As shown in Fig. 1B, treatment with either the diacylglycerol analog OAG or AA was effective at stimulating ROS production in CF. The possibility that OAG and AA may stimulate PKC, and sequentially activate Nox, through a phorbol ester-independent mechanism was investigated by pretreatment with the specific PKC inhibitor bisindolemaleimide (BIM) and pan-kinase inhibitor

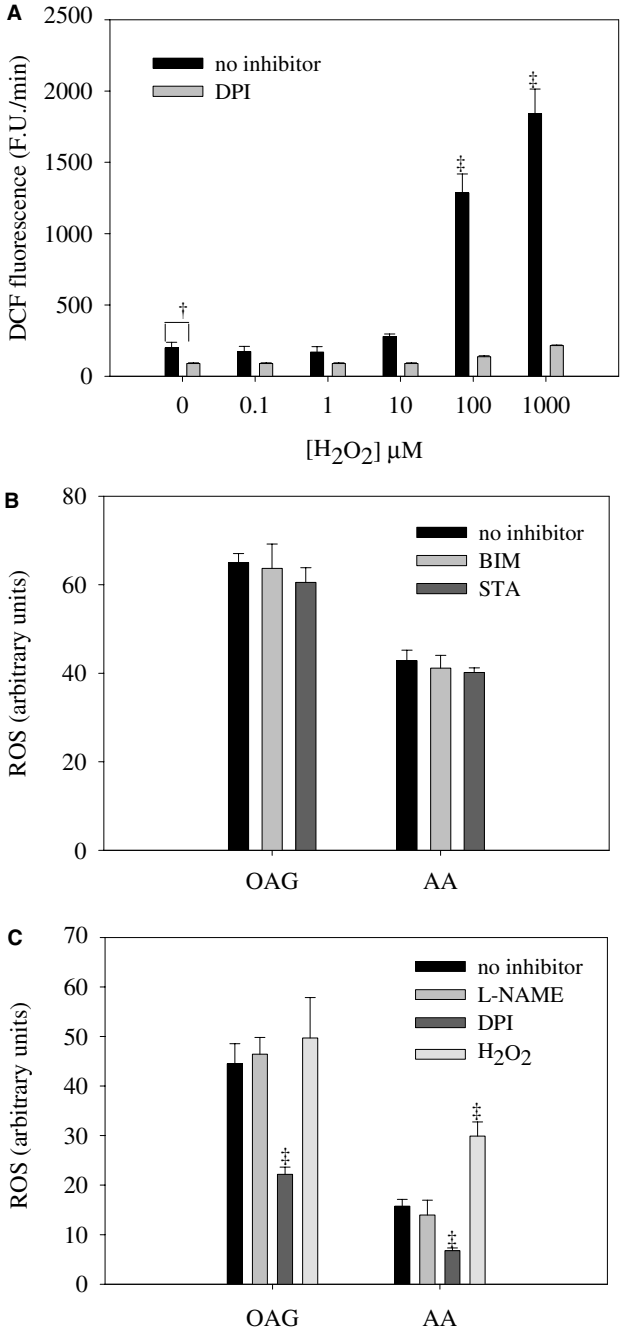

Fig. 1. Panel A, brief H_2O_2 stimulates ROS generation in CF. DCF-loaded primary CF were stimulated as indicated and monitored for 1 h in a microplate. Values represent means ± 1 S.D. ($n = 6$); $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ versus no H_2O_2 stimulus. Panel B, effects of kinase inhibitors on OAG- and AA-stimulated ROS generation. DCF-loaded primary CF were stimulated with the diacylglycerol analog OAG (100 μM) or AA (100 μM) in the absence (no inhibitor) or presence of the following kinase inhibitors: BIM (400 nM) and STA (50 nM). DCF fluorescence (ROS) was monitored for 1 h with kinetic data normalized to cell count by CyQuant assay as described under Section 2. Values represent means ± 1 S.D. ($n = 6$). Panel C, exogenous H_2O_2 potentiates AA- but not OAG-stimulated ROS generation in CF. DCF-loaded primary CF were stimulated with either OAG (100 μM) or AA (100 μM) and monitored for 1 h in a microplate fluorometer. Data was normalized to cell count by CyQuant assay as described under Section 2. Values represent means ± 1 S.D. ($n = 6$); $^{\ddagger}P < 0.001$ versus no inhibitor.

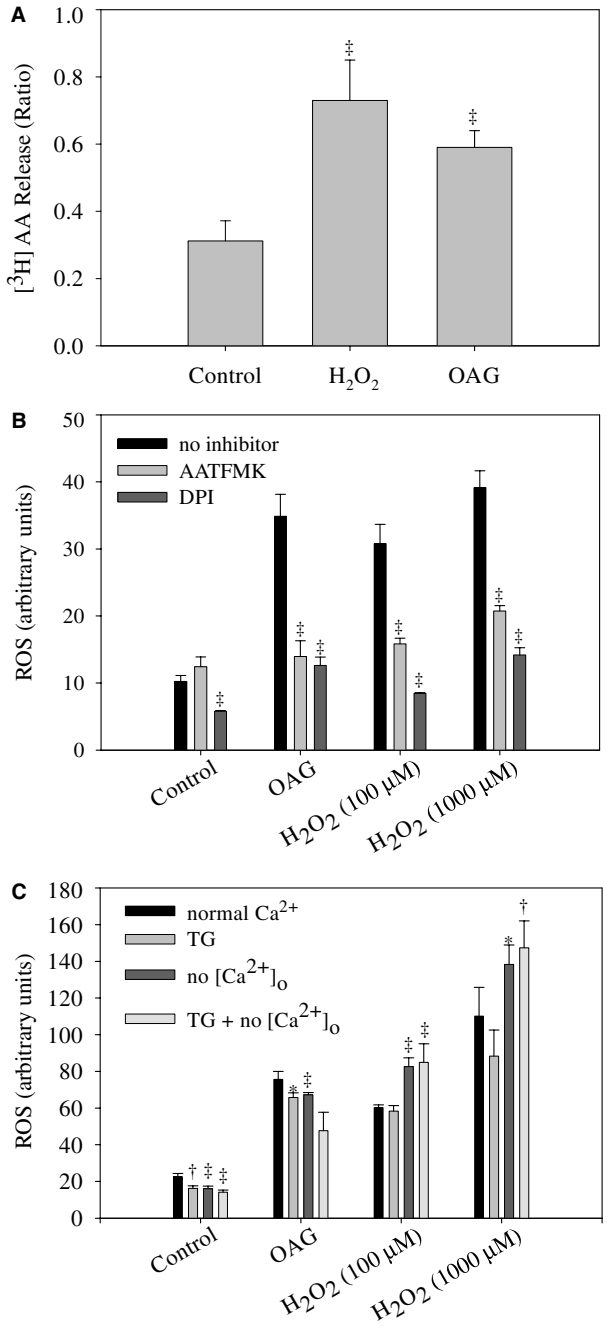

Fig. 2. Panel A, treatment of CF with brief H_2O_2 (100 μM) or OAG (100 μM) stimulates [^{3}H]AA release. Primary CF (passage 2) were labeled overnight with 0.5 mCi [3H]AA, washed and stimulated for 1 h as indicated. [^{3}H]AA release was determined by scintillation counting. Values represent means ± 1 S.D. ($n = 6$); $^{\ddagger}P < 0.001$ versus no stimulation and normalized to DNA content (CyQuant). Panel B, H_2O_2 and OAG stimulate ROS generation through a PLA_2-dependent mechanism. DCF-loaded primary CF were stimulated with H_2O_2 or OAG in the presence or absence of the PLA_2 inhibitor AATFMK (100 μM) or DPI. Values represent means ± 1 S.D. ($n = 6$); $^{\ddagger}P < 0.001$ versus no inhibitor.Panel C, role of Ca^{2+} in H_2O_2- and OAG-stimulated ROS generation. DCF-loaded primary CF were stimulated with OAG (100 μM) or H_2O_2 under the following conditions: 1.8 mM Ca^{2+} (normal Ca^{2+}), intracellular Ca^{2+} depletion following thapsigargin pretreatment (TG), no extracellular Ca^{2+} (no $[Ca^{2+}]_o$), and intracellular Ca^{2+} depletion with no extracellular Ca^{2+} (TG + no $[Ca^{2+}]_o$). Data was normalized to cell count by CyQuant assay as described under Section 2. Values represent means ± 1 S.D. ($n = 6$); $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ versus normal Ca^{2+}.

staurosporine (STA). Fig. 1B shows the minimal effects these inhibitors had on OAG- and AA-stimulated ROS generation. Similar to H_2O_2, Fig. 1C demonstrates that OAG- and AA-stimulated ROS production was sensitive to DPI but not L-NAME suggesting peroxide, OAG, and AA may target a common ROS generating complex. Co-stimulation with OAG and H_2O_2 together did not significantly increase ROS production as compared to OAG alone whereas stimulation with AA and H_2O_2 together had additive effects. These data taken together suggest H_2O_2 and OAG may stimulate ROS production through a common, rate-limited pathway whereas exogenous AA stimulates ROS production through a parallel pathway, though all appear to ultimately activate a common, DPI-sensitive oxidase.

OAG and H_2O_2 are known activators of phospholipase A_2 (PLA_2) leading to phospholipid turnover and AA release, which has been shown to activate Nox in some cells [20,21]. Therefore, we investigated whether PLA_2 activation by peroxide or OAG could be central to subsequent ROS generation. As shown in Fig. 2A, stimulation with either brief H_2O_2 or OAG was effective at stimulating AA release from primary CF. Fig. 1B shows the effects of $cPLA_2$ inhibition on OAG- and H_2O_2-stimulated ROS production. Pretreatment with the PLA_2 inhibitor, arachidonyl trifluoromethyl ketone (AATFMK; 100 μM) significantly blunted OAG and peroxide stimulated ROS. By comparison, DPI was slightly more potent as an inhibitor of ROS production than AATFMK. These data support the hypothesis that $cPLA_2$ is the primary target of OAG and peroxide and AA may be the common mediator leading to Nox activation in primary CF. However, whether other DPI-sensitive oxidases are also activated and contribute to the observed ROS signals is unknown and will require further investigation.

Since endogenous ROS generation may be redox sensitive [22,23], we studied the effects of various antioxidants including diethyldithiocarbamate (DDC), pyrrolidinedithiocarbamate (PDTC), *N*-acetyl cysteine (NAC), and the combination of ascorbate and α-tocopherol (Vitamins C/E). Table 2 shows that while all antioxidants reduced ROS generation, DDC was the most potent antioxidant reducing DCF fluorescence to background levels independent of the stimulus. NAC and PDTC were somewhat less potent and vitamins C/E were the least effective at attenuating ROS generation. Experiments were conducted with antioxidant pretreatments (while DCF loading) and with antioxidants administered simultaneously with the stimulant (after DCF loading). In each case, whether the cells were pretreated with antioxidants or treated simultaneously with H_2O_2, OAG, or AA, the ROS quenching effects were unaltered indicating antioxidants did not affect DCF loading.

We have previously shown [1] that exposure of CF to brief H_2O_2 results in large Ca^{2+} fluxes which were shown subsequently to be inhibited by DPI. Although the ROS-dependence of stimulated Ca^{2+} fluxes was demonstrated unambiguously, whether ROS generation in CF requires Ca^{2+} fluxes is unknown. This was investigated by stimulating cells with OAG and H_2O_2 (i) in buffer containing normal Ca^{2+} (1.8 mM; no treatment), (ii) in buffer containing normal Ca^{2+} where intracellular Ca^{2+} was depleted with thapsigargin (TG), (iii) in Ca^{2+}-free buffer without thapsigargin (no $[Ca^{2+}]_o$), and (iv) by stimulating Ca^{2+}-depleted cells in Ca^{2+}-free buffer. Fig. 2C demonstrates that reducing or eliminating Ca^{2+} fluxes does

Table 2
Antioxidants inhibit stimulated ROS generation in CF

	No inhibitor	DDC	PDTC	NAC	Vitamins C/E
Control	10.7 ± 0.5	2.0 ± 0.3‡	5.4 ± 0.4‡	5.9 ± 0.9‡	3.5 ± 0.4‡
OAG	49.8 ± 5.2	2.7 ± 0.4‡	18.3 ± 1.7‡	26.2 ± 4.8†	28.1 ± 2.9‡
AA	35.3 ± 2.0	1.6 ± 0.4‡	N.D.	15.3 ± 2.3‡	9.28 ± 0.7‡
H_2O_2 (100)	44.3 ± 2.4	3.1 ± 0.5‡	20.2 ± 2.1‡	11.5 ± 1.7‡	30.0 ± 3.2†
H_2O_2 (1000)	69.9 ± 3.0	3.0 ± 0.2‡	28.4 ± 3.0‡	24.9 ± 7.8‡	44.9 ± 1.6‡

Abbreviations: DDC, diethyldithiocarbamate; PDTC, pyrrolidinedithiocarbamate; NAC, *N*-acetyl cysteine; vitamin C, ascorbate; E, α-tocopherol. †$P < 0.01$, ‡$P < 0.001$ (versus no inhibitor), N.D. not determined.

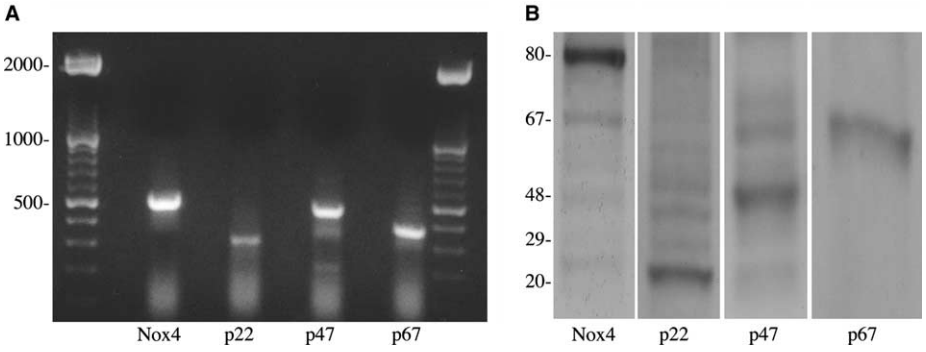

Fig. 3. Nox4 and phox subunit expression in primary CF. Total RNA was isolated, reverse transcribed and amplified by PCR (35 cycles) using the primers shown in Table 1. Panel A shows Nox4, p22, p47, and p67 amplicons visualized on an ethidium bromide-stained 2% agarose gel. (B) Total protein was isolated from primary CF, separated on a 12% SDS–PAGE gel, blotted onto nitrocellulose and detected by enhanced chemiluminescence.

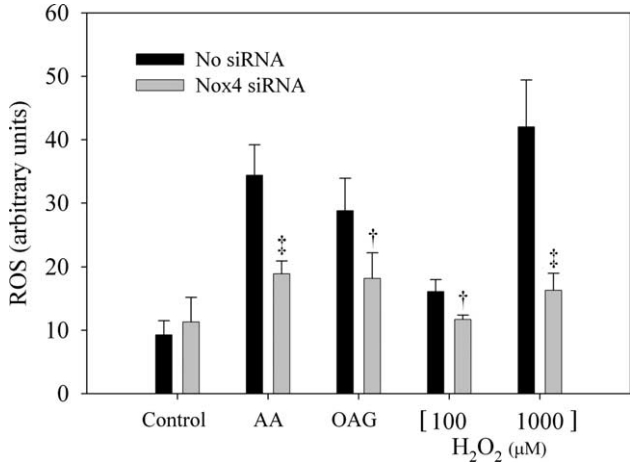

Fig. 4. Nox4 siRNAs blunts stimulated ROS generation in CF. Primary CF grown to 50% confluence in 100 mm plates were transfected with Nox4 siRNA and stimulated with OAG, AA, and H_2O_2 after 48 h. Data represent means ± 1 S.D. ($n = 6$). †$P < 0.01$, ‡$P < 0.001$ versus no siRNA.

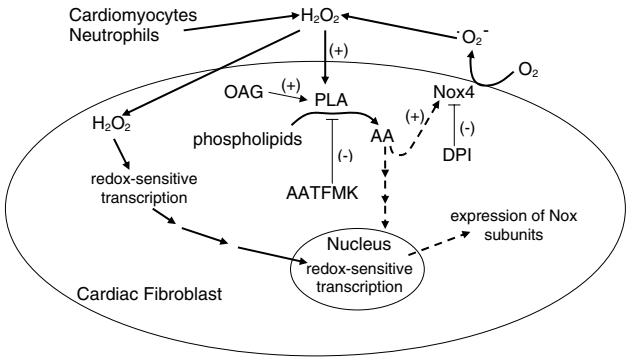

Fig. 5. Oxidant-induced activation of CF Nox4. Exogenously generated H_2O_2 activates CF PLA_2 leading to AA release and activation of Nox4. Activation of Nox4 by AA may occur directly and/or through induction of Nox subunits (dashed lines). Nox4 generates superoxide which is converted to H_2O_2 either enzymatically (dismutase) or spontaneously resulting in redox-sensitive gene transcription. AATFMK, arachidonyl trifluoromethyl ketone; DPI, diphenylene iodonium; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol.

not inhibit the bulk of ROS generation and, in the case of peroxide stimulation, may actually potentiate ROS production.

RT-PCR and Western blotting were used to detect phox subunits to define potential elements of a functional Nox complex in CF (Fig. 3). RT-PCR primers were designed to amplify all known phox subunits. In cases where negative results were obtained, a second, and in some cases a third primer pair was employed. Results show (Panel A) that unstimulated primary CF express mRNA transcripts for Nox4, p22 phox, p47 phox, and p67 phox. These results were confirmed by Western blotting and are shown in Fig. 3B. The Nox4 polyclonal antibody detected a dominant band of approximately 80 kDa, slightly larger than the 75 kDA predicted size [4]. Bands corresponding to p22 phox, p47 phox, and p67 phox were similar to expected molecular weights. We attemped to block Nox4 activity by (i) transfecting cells with p22 phox antisense oligonucleotides and

(ii) by transfecting cells with siRNAs directed aginst the Nox4 transcript. Treatment with p22 antisense oligos significantly reduced the rate of peroxide-stimulated ROS generation by approximately 50%, from 526 ± 31 F.U./min to 271 ± 25 F.U./min ($P < 0.01$; no oligo versus p22 phox antisense, respectively) similar to earlier reports [24,25]. Transfections with scrambled and sense oligos had minimal effects on rates of stimulated ROS generation (not shown). Fig. 4 demonstrates that CF transfected with Nox4 siRNA resulted in significant reductions in OAG-, AA-, and H_2O_2-stimulated ROS generation. The inability of p22 phox antisense and Nox4 siRNA treatments to completely block stimulated ROS production may be due to incomplete inhibition of the expression of these gene products or may suggest the presence of other DPI-sensitive ROS generating mechanisms in adult CF. These data demonstrate that CF express a functional cytochrome b_{558} with the catalytic core likely composed of Nox4 and p22. Whether p47 phox, p67 phox, or other protein(s) are also required for ROS generating activity is unknown and will require further investigation. A proposed scheme showing oxidant-induced Nox4 activation in CF is presented in Fig. 5.

4. Discussion

This study demonstrates for the first time that adult CF express an ROS-inducible, PLA_2-dependent NADPH oxidase that is rapidly activated by H_2O_2, diacylglycerol (OAG), and AA but is insensitive to phorbol esters and elevated intracellular Ca^{2+}. RT-PCR and Western blotting demonstrate that primary adult CF express abundant Nox4, p22, p47, and p67 phox mRNA and proteins. We observed that CF also express very weak Nox2 mRNA by RT-PCR but protein was undetectable by Western blotting. Furthermore, stimulated ROS generation was sensitive to antioxidants and the flavin-containing enzyme inhibitor DPI but does not appear to involve significant contributions from nitric oxide synthase (NOS) activity as inhibitors of NOS were without effect, and nitric oxide (NO) metabolites were undetectable in stimulated culture supernatants. Our results suggest that CF express an ROS generating system that may be unique compared to other fibroblasts and that Nox expression and function in CF is tissue specific.

Recent work has demonstrated that adventitial fibroblasts generate extracellular superoxide following activation of Nox2 (formerly gp91 phox) [26]. Expression of the phagocytic-type Nox2 enzyme has been reported in fibroblasts from various tissues [26–28]. Wang et al. [26] showed that aortic advential cells express Nox2, p22, p47, and p67 phox subunits. These authors demonstrated that superoxide production by these cells was not affected by inhibition of other ROS producing enzymes including xanthine oxidase, mitochondrial oxidases, and NOS. These data are supported by Sorescu et al. [27] who showed Nox2 immunoreactivity mainly in the adventitia whereas Nox4 was abundantly expressed in the smooth muscle cells of the media and only weakly in fibroblasts. Adventitial fibroblasts play an active role in regulating vascular tone by limiting the diffusion and bioavailability of NO; superoxide inactivates NO by reacting to produce peroxynitrite [29]. In contrast, we show that primary CF mainly express Nox4 and not Nox2 indicating that fibroblast Nox expression is tissue specific. Such differences in Nox expression suggests there may be important and unique functions of fibroblasts that reside in diverse tissues.

Nox4 (formerly RENOX) expression has been demonstrated primarily in kidney, uterus, and testes [8] and has been suggested to mediate oxygen sensing [30] in some tissues. The presence of Nox4 expression in CF supports the hypothesis that fibroblasts, in addition to regulating extracellular matrix, may function as oxygen sensors in the heart. Ultrastructural work by Nag [31] and others have shown that 65–70% of cells in the mammalian heart are non-myocytes. The non-myocytes were subsequently shown to be >90% CF and were observed in close contact with myocytes and vascular structures [32]. We [1] and others [2,33,34] have demonstrated that CF constitute a rich source of cytokines, chemokines, and growth factors, substances with known effects on myocyte function. For example, stimulation of CF with a brief pulse of physiological levels of H_2O_2 results in transient activation of MAP kinase pathways and induction of IL-6 [1], a cytokine with hypertrophic and anti-apoptotic properties in the heart. Thus, given the abundance of CF in the heart and their close proximity to working myocytes and vascular cells, it is likely that CF play an important role in sensing changes in the physical and biochemical milieu and produce paracrine factors capable of modulating myocyte function. Our investigations suggest ROS generation by CF could participate in the process by which myocytes adapt to stress.

The molecular structure and activation characteristics of Nox4 are poorly understood. Gorin et al. [35] have recently reported the expression and angiotensin II (AII)-induced activation of Nox4 in mesangial cells. These authors showed that binding of AII to its G protein-coupled receptor results in activation of PLA_2, AA production, Nox4 activation and ROS generation. The Nox4 expressed in CF shares similarities with the mesangial cell oxidase but significant differences in activation were observed. We found that inhibition of $cPLA_2$ with AATFMK significantly reduced ROS generation following brief stimulation with H_2O_2 or the diacylglycerol analog OAG, and that this inhibitor was of similar magnitude as was seen with DPI pretreatment (see Fig. 2B). Phorbol esters and diacylglycerols are potent activators of several Nox isoforms including phagocytic Nox2 which may [36] or may not [20] require PKC activity. We found that CF Nox activation does not require PKC. Peroxide-, OAG-, and AA-stimulated ROS generation in CF was insensitive to both phorbol esters (PMA) and PKC inhibition with BIM or STA (see Fig. 1B). In this regard, diacylglycerols are known to bind and activate enzymes other than PKCs including phospholipases (e.g., $cPLA_2$) [37]. Some phospholipases are also activated by ROS including H_2O_2 [38,39] providing a common signaling pathway for DAGs and ROS. Further evidence supporting a central role for PLA_2 is provided by the observation that OAG-stimulated ROS generation was not potentiated by exogenous H_2O_2 whereas it was additive when administered with AA (see Fig. 1C). These data suggest that both OAG and H_2O_2 stimulate ROS production through a shared, rate-limited enzymatic pathway likely involving $cPLA_2$ and implicate AA as a common signaling molecule leading to Nox activation in CF.

A simple yet plausible mechanism for stimulated CF Nox activity involves the sequential activation of PLA_2, AA production and ROS generation (Fig. 5). Whether AA directly

activates Nox or acts as an intermediate signaling molecule in CF is unknown. Current understanding of Nox2 activation by AA in neutrophils suggests a role for calmodulin and/or calmodulin-binding proteins [40]. Our data show that depletion of intracellular Ca^{2+} had no significant effect on ROS generation, whereas stimulation of Ca^{2+}-depleted CF in Ca^{2+}-free buffer actually potentiated ROS. These data demonstrate AA-stimulated Nox activity may involve Ca^{2+}-dependent or -independent mechanisms dictated by the isoform and/or subunit composition of the cytochrome b_{558} complex and/or the cell type and tissue.

RT-PCR and Western blotting demonstrate that CF express mRNA transcripts and proteins for Nox4, p22, p47, and p67 phox. Although a detectable RT-PCR product was observed for Nox2, we could not detect Nox2 protein by Western blotting. Other phox subunits including p40, p41, and p51 could not be detected by RT-PCR using multiple primers. In order to determine whether the Nox4/p22 complex was indeed the major oxidase contributing to stimulated ROS production in CF, we transfected the cells with Nox4 siRNA and p22 antisense oligonucleotides and assayed for basal and stimulated ROS generation after 48 h. Treatment with p22 antisense oligos reduced the rate of H_2O_2 stimulated ROS generation by >50% which is in agreement with other reports [24,25]. Sense and scrambled oligos had minimal effects. Similarly, as shown in Fig. 7, transfection of CF with a combination of two Nox4 siRNAs resulted in significant reductions in OAG, AA, and H_2O_2-stimulated ROS generation after 48 h, similar to other reports [41]. A role for p47 phox and p67 phox in stimulated CF ROS production was not determined in the present study and will require further investigation.

We report that primary CF express an ROS-, diacylglycerol- and AA-stimulated oxidase that is rapidy activated and insensitive to phorbol esters and PKC inhibition. The major NADPH oxidase isoform expressed by CF is composed of Nox4 and p22 phox proteins. Though other phox subunits were detected, their function in the CF oxidase complex remains unclear and will required further studies. This oxidase was shown to be activated in a dose-dependent manner by H_2O_2 and diacylglycerol which sequentially stimulate $cPLA_2$ activity, generate AA, and activate Nox4 leading to intracellular ROS production. The ROS generation was inhibitable to various degrees following pretreatments with antioxidants or DPI. In conclusion, we show for the first time that CF express mRNA and protein for Nox4, p22 phox, p47 phox, and p67 phox implicating this enzyme complex in stimulated ROS generation in CF.

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