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Activation of the EGFR/p38/JNK Pathway by Mitochondrial-Derived Hydrogen Peroxide Contributes To Oxygen-induced Contraction Of Ductus Arteriosus

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Abstract

Oxygen-induced contraction of the ductus arteriosus (DA) involves a mitochondrial oxygen-sensor, which signals pO_2 in the DA smooth muscle cell (DASMC) by increasing production of diffusible hydrogen peroxide (H_2O_2). H_2O_2 stimulates vasoconstriction by regulating ion channels and rho kinase, leading to calcium influx and calcium sensitization. Because epidermal growth factor receptor (EGFR) signaling is also redox regulated and participates in oxygen sensing and vasoconstriction in other systems, we explored the role of the EGFR and its signaling cascade (p38 and JNK) in DA contraction.

Experiments were performed in DA rings isolated from full-term New Zealand White rabbits and human DASMC. In human DASMCs increasing pO_2 from hypoxia to normoxia (40 to 100 mmHg) significantly increased cytosolic calcium, $p<0.01$. This normoxic rise in intracellular calcium was mimicked by EGF and inhibited by EGFR siRNA. In DA rings, EGF caused contraction whilst the specific EGFR inhibitor (AG1478) and the tyrosine kinase inhibitors (genistein or tyrphostin A23) selectively attenuated oxygen-induced contraction ($p <0.01$). Conversely, orthovanadate, a tyrosine phosphatase inhibitor known to activate EGFR signaling, caused dose-dependent contraction of hypoxic DA and superimposed increases in oxygen caused minimal additional contraction. Ansomycin, an activator of EGFR's downstream kinases, p38 and JNK, caused DA contraction; conversely, oxygen-induced DA contraction was blocked by inhibitors of p38 MAPK (SB203580) or JNK (JNK inhibitor II). O_2-induced phosphorylation of EGFR occurred within 5-minutes of increasing pO_2 and was inhibited by mitochondrial-targeted overexpression of catalase. AG1478 prevented the oxygen-induced p38 and JNK phosphorylation. In conclusion, O_2-induced EGFR transactivation initiates p38/JNK-mediated increases in cytosolic calcium and contributes to DA contraction. The EGFR/p38/JNK pathway is regulated by

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mitochondrial redox signaling and is a promising therapeutic target for modulation of the patent ductus arteriosus.

Keywords

Oxygen sensing; tyrosine kinase inhibitors; c-Jun N-amino-terminal kinase (JNK); Patent ductus arteriosus (PDA); protein tyrosine phosphatases; Mitochondrial hydrogen peroxide

Introduction

The ductus arteriosus (DA) plays an essential role in fetal life, diverting blood away from the unventilated lungs. At birth, the increase in arterial pO_2 causes DA contraction, thus diverting blood flow to the newly expanded lungs. Although vasodilatory effects of prostaglandin E2 and constrictor substances such as endothelin-1[1] modulate this contraction, oxygen-induced contraction is an intrinsic property of DA smooth muscle (DASMC)[2; 3]. Persistent patent ductus arteriosus (PDA) is a common abnormality in the newborn, affecting 55% of low birth weight, premature infants[4], resulting in heart failure, and failure to thrive. Conversely, in some congenital heart disease conditions, it is critical to maintain DA patency to provide pulmonary or systemic blood flow for the newborn. Thus, understanding the mechanism by which O_2 constricts the DA is an important physiologic question and has practical translational applications in the treatment of PDA.

Rapid increases in pO_2 in DASMC are detected by a mitochondrial redox sensor. In response to a physiologic rise in pO_2, the DASMC's mitochondria elaborate more reactive oxygen species (ROS), including H_2O_2[5; 6]. These ROS constitute a redox signal and initiate several vasoconstrictive events, including: (1) inhibition of the redox-sensitive, voltage-gated, potassium channels, resulting in membrane depolarization and calcium entry through large conductance calcium (L-type) channels[3], (2) direct redox-mediated activation of L-type calcium channels[7], (3) release of calcium from the sarcoplasmic reticulum and activation of store-operated channels[8; 9], and (4) calcium-sensitization, primarily reflecting Rho kinase activation[8; 9; 10].

Mitochondrial-derived H_2O_2 production is dynamically modulated in proportion to pO_2[5] and is involved in initiating DASMC contraction[11]. H_2O_2 is a diffusible signal that changes vascular tone by oxidizing sulfhydryl moieties in amino acids, such as cysteine, that regulate the conformation and function of enzymes and ion channels. By oxidizing catalytic cysteine residues H_2O_2 also inactivates protein-tyrosine phosphatases, lipid phosphatases and tumor suppressor phosphatase and tension homolog (PTEN), which modulates protein phosphorylation[12]. Tyrosine phosphorylation regulates many cellular responses, including contraction[13; 14]. In vivo, tyrosine phosphorylation is reversible and dynamic. The phosphorylation states are controlled by the coordinated action of protein tyrosine kinases (PTK) and phosphatases (PTP). Phosphorylation of tyrosine residues is involved in Ca^{2+} influx, intracellular Ca^{2+} release, and Ca^{2+} sensitization of the contractile apparatus[15]. Clyman et al have reported that the tyrosine kinase inhibitor, genistein (100 μM) inhibited both Ca^{2+} -dependent and Ca^{2+}-independent contraction in DA rings[9]; however, the specific tyrosine kinase involved was not identified.

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. They are transmembrane proteins that are activated upon binding peptide growth factors and are involved in cellular transformation in cancer[16]. EGFR inhibitors, such as erlotinib, are used clinically to treat various cancers[17]. In the A431 cancer cell line, hypoxia activates EGFR signaling and induces epithelial to mesenchymal transition[18]. Hypoxia prolongs EGFR activation through an hypoxia-inducible factor-dependent deceleration of endocytosis-mediated EGFR deactivation[19].

Although there is little information regarding the role of EGFR in vascular oxygen sensing, EGFR transactivation augments depolarization-induced Ca^{2+} sensitization and vasoconstriction in chronically hypoxic pulmonary vascular SMC[20]. Moreover, EGFR transactivation is involved in H_2O_{2-}, endothelin-1-, and angiotensin II-signaling [21; 22; 23]. EGFR mediates its effects by activation of a downstream kinase cascade that includes mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERKs), c-Jun N-amino-terminal/stress-activated protein kinases (JNKs/SAPKs), and p38 MAPKs[21; 24; 25].

We hypothesize that oxygen-induced increases in mitochondrial H_2O_2 activate EGFR and contributes to oxygen-induced DA contraction. We investigated whether oxygen activates EGFR and used pharmacological probes to determine its downstream signaling cascade in rabbit DA rings and human DASMC. We report that that H_2O_2-dependent EGFR activation raises cytosolic calcium and promotes DA contraction by increasing p38/JNK signaling. EGFR inhibition reduces constriction to oxygen but not KCl. We conclude that the phosphorylation status of EGFR is redox sensitive and is a key regulator of oxygen sensing in the ductus arteriosus.

Methods

All animal studies were approved by the Institutional Animal Care and Use Committee of the Minneapolis Veterans Affairs Medical Center and The University of Chicago Animal Care Committee and conform to current National Institutes of Health and American Physiology Society guidelines for the use and care of laboratory animals. Human DAs were obtained from infants with various forms of congenital heart disease at the time of surgical correction either at the University of Nebraska Children's Hospital or the University of Chicago, under protocols approved by the local institutional review board of both institutions. The demographics of these children, none of whom had PDA, has previosuly been described[5; 6].

DA rings were isolated from pregnant New Zealand white rabbits at 30 –31 days of gestation (term), as previously described[6; 8]. Briefly, the rabbits were anesthetized with ketamine (75 mg) and xylazine (20 mg) intramuscularly and pentobarbital (50 mg) intravenously. The fetal pups were delivered by cesarean section and a midline sternotomy was performed, before initiation of respiration. The heart, lungs, and great vessels were excised en bloc and placed in deoxygenated Earle's solution. The DA was carefully dissected free from adventitia under a dissecting microscope and then excised, excluding its attachments to the left pulmonary artery and descending thoracic aorta.

DA ring tension measurement

The isolated rabbit DA ring was connected to strain gauge transducers by two stainless steel wires. The ring was suspended in a 3-ml bath containing Earle's solution. The ring was equilibrated with an hypoxic gas mixture (0 % O_2, 5 % CO_2; pO_2 = 22 ±1 mmHg). The optimal resting tension (1000 mg) was determined in hypoxic medium by measuring the maximum contractile response to KCl (60 mM) at varying basal tensions (200–2,000 mg). Indomethacin (3 μM) and L-NAME (100 μM) were present throughout all experiments. O_2 tension was recorded continuously with an M1-730 oxygen electrode (Microelectrode, Bedford, NH). A normoxic gas mixture (20% O_2, 5% CO_2, pO_2=133 ±1 mmHg) was used to bubble the chamber solution creating normoxia. Data were recorded using an analog-digital computer system (MatLab, AD Instruments Inc., Medford, MA).

Human DASMC culture

DASMCs from human neonates were isolated from freshly isolated human ductus arteriosus. The identity of the cells as DASMCs was confirmed by confirming positive immunostaining for SM α-actin but not vonWillebrand's factor. A primary culture of DASMCs was established and then the cells were harvested with trypsin, frozen in Freezing Media (10% DMSO, 50% FBS and 40% DMEM) and stored in liquid nitrogen for later use. DASMC were grown in 100 mm culture dishes and used within the first 5 passages in culture. DASMC were maintained in hypoxia (pO_2 40 mmHg, pH 7.35–7.45, pCO_2 30–40 mmHg using an environmentally controlled, Tri-Gas CO_2 incubator (Thermo Scientific) until the protocol called for exposure to normoxia.

Exposure of hypoxic human DASMC to acute normoxia

The experiment was performed in a chamber flushed with nitrogen. Inside the hypoxic chamber, 2 small glass containers (500 mL) were filled with Earle's solution (37°C). They were bubbled either with an hypoxic (0% O_2, 5% CO_2; pO_2=28 ± 2 mmHg) or normoxic (20% O_2, 5% CO_2; pO_2=134 ± 2 mmHg) gas mixture. DASMCs were immersed in the hypoxic solution for 30 minutes and then either maintained in hypoxia for 1, 3, 5, 10, or 20 minutes or quickly switched to the normoxia solution for 1, 3, 5, 10, or 20 minutes. At the end of the experiment, cells were flash frozen in liquid nitrogen and later lysed at 4°C under hypoxic conditions using the phosphoSafe extraction reagent (Calbiochem, Billerica, MA) and a phosphatase inhibitor cocktail (Calbiochem, 1:100, Billerica, MA).

Real-time PCR

Total RNA was isolated from cultured cells or harvested tissues using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). Total RNA (0.5 μg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Equal volumes of the resultant cDNA were used to evaluate the expression level of mRNAs among treatments and controls by real-time PCR. The quantitative real-time PCR was performed on an Applied Biosystems 7900HT (Life Technologies, Grand Island, NY) as previously described[6]. The primers used were purchased from Applied Biosystems (Probe ID: EGFR: Hs01076087_m1; 18S: Hs99999901_s1).

SiRNA for EGFR

To achieve specific molecular knockdown of EGFR, we used several small interfering RNA (siRNA) as previously described[6]. SiRNA for EGFR were: 5′GGCUGGUUA UGUCCUCAUUGCCCTC-3′; 5′GAGGGCAAUGAGGACAUAACCAGCCAC-3′; 5′-GGCG GGACAUAGUCAGCAGUGACTT-3′ and 5′-AAGUCACUGCUGACUAUGUCCCGCCAC-3′. A scrambled siRNA control was used: 5′-CUUCCUCUCUUUCUCUCCCUUGUGA-3′. siRNA was purchased from Integrated DNA Technologies (Coralville, IA, United States).

Immunoblotting

Equal amounts of protein (20 μg) were subjected to SDS/PAGE gradient gels (4–15%), transferred to polyvinylidene difluoride membranes, blocked with 5 % (w/v) BSA in Tris buffered saline with Tween 20 (TBST; 25 mM Tris-HCl, pH 7.4, 137 mM NaCl and 0.1 % Tween-20) for 1-hour, and incubated with primary antibodies in 5 % (w/v) BSA in TBST for 2-hours at room temperature. The membranes were washed at least three times with TBST at 15-minute intervals and then incubated with either mouse or rabbit or goat horseradish peroxidase-conjugated secondary antibody (1:2000) for 1-hour at room temperature. The membranes were developed with an enhanced chemiluminescence detection system according to the manufacturer's instructions. The following antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX): rabbit anti-EGFR (sc-003, used for immunoblotting), mouse anti-L-caldesmon (1:200), goat anti- phospho –caldesmon (tyr27, 1:400). The following antibodies were from Cell Signaling Technology, Inc. (Boston, MA): rabbit anti-p38 MAPK (1:1000), rabbit anti-phospho-p38 MAPK (thr180/tyr182, 1:1000), rabbit anti- SAPK/JNK (1:1000), rabbit anti-phospho-SAPK/JNK (thr183/tyr185, 1:1000). The following antibodies were from EMD (Gibbstown, NJ): ST1030 PhosphoDetect Anti-Hsp27 (pSer82) mouse mAb (1:400), mouse anti-Hsp27 (1:1000). Mouse anti-caldesmon was from Sigma (1:1000).

Immunoprecipitation

A total of 400 μg of protein from human DASMCs was used for immunoprecipitation with mouse anti-EGFR monoclonal antibody (IgG2a, sc-120). Following gel electrophoresis, tyrosine-phosphorylated EGFR was detected by immunoblotting with monoclonal anti-phosphotyrosine (SC-508, Santa Cruz biotechnology, Dallas, TX). Total EGFR was analyzed by re-probing of the same filter with anti-EGFR antibody (sc-003, 1:400, Santa Cruz biotechnology, Dallas, TX).

Calcium imaging

Dual-excitation imaging with fura-2 was used to measure the cytosolic Ca^{2+} response to changes in oxygen tension in cultured human DASMC, as described[6; 26]. The human DASMC were transferred to imaging dishes (Molecular Probes, Eugene, OR), and incubated in low-Ca^{2+} Hanks' solution with the cell-permeable acetoxymethyl ester form of fura 2 (0.1 μM) and Pluronic F-127 (0.8 μM) for 15 minutes at room temperature. The plates were then washed with HEPES buffer containing 1.5 mM Ca^{2+} and incubated at room temperature for a further 20 minutes. The plates were then washed again and placed on the microscope

stage. The drugs were added directly to the cells as a bolus by microinjection. All drugs were given in 10 μl volumes to reduce potential volume-induced artifacts. Saline injections of 10 μl had no effect on $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were recorded in individual cells using a 340/380 nm MetaFluor filter-based imaging system (Universal Imaging, West Chester, PA) and a cooled charge-coupled device camera (Photometrics, Tucson, AZ).

Statistical Analysis

Data are expressed as mean±SEM. In all figures, the SEM is indicated, although in some cases it did not exceed the symbol size. Differences between groups were calculated using an unpaired Student's t-test or simple ANOVA with post hoc analysis (Fisher's least-significant-difference test), as appropriate. A $p<0.05$ was considered statistically significant.

Results

Gs proteins are involved in O_2-induced DA contraction

EGFR is known to signal via G-proteins[27]. Consequently we assessed the effects of cholera toxin (a Gs inhibitor that is known to block the effects of EGFR[28]) and pertussis toxin (a Gi inhibitor) on O_2-induced contraction. Cholera toxin (1 mg/ml, 20–60 minutes incubation) attenuated the O_2-induced DA contraction by ~ 80% (supplemental fig. 1 A–B) without affecting the hypoxic baseline tone or the contraction to KCl (60 mM), data not shown. Pertussis toxin (1 mg/ml, administered 20–60 minutes prior to increasing pO_2) had no effect on O_2-induced DA contraction (supplemental fig. 1C). Because substantial published evidence suggests that endothelin may also contribute to DA contraction, we assessed the effects of endothelin receptor antagonists on DA contraction to oxygen[29]. BQ-123 (selective ET_A receptor antagonist, 3 μM) and BQ-788 (selective ET_B receptor antagonist, 3 μM) minimally reduced O_2-induced contraction (supplemental fig. 1D), consistent with our previous findings[30]. These findings support the contention that oxygen-induced DA contraction involves Gs proteins and is not primarily dependent on endothelin-1.

Tyrosine kinases and tyrosine phosphatases are involved in O_2-induced DA contraction

Two structurally and functionally distinct tyrosine kinase inhibitors, genistein (fig. 1A and 1C) and tyrphostin A23 (fig. 1B and 1C), markedly reduced O_2-induced DA contraction. Daidzein, an inactive control for genistein had no effect on O_2-induced DA contraction (fig. 1C). We next assessed whether tyrosine kinase inhibition targeted the upstream oxygen sensing or just the downstream effectors (activation of L-type calcium channels or store operated channels, SOCs). Genistein had no effect on 60 mM KCl-induced contraction (Fig 1D). This suggests that genistein affects an oxygen-sensing mechanism in the DA, rather than acting directly on downstream regulators of membrane potential, such as voltage-gated (Kv) channels, or L-type calcium channels (which together mediate KCl-induced contraction). Since SOCs have been implicated in DA contraction, we evaluated the possible effects of tyrosine kinase on SOCs by exposing DA rings to cyclopiazonic acid (CPA) in a calcium-free bath solution, to empty intracellular calcium and activate SOCs. A subsequent switch of the extracellular calcium from 0 to 2 mM (in the presence of nifedipine to block voltage-gated calcium channels) induced contraction, reflecting calcium entry through

SOCs[8]. The finding that SOC-mediated DA contraction was also unaffected by genistein (fig. 1E) indicates that the SOCs are not direct targets of tyrosine kinase in the DA.

The selective inhibitory effects of genistein on oxygen-induced contraction but not on contraction resulting from membrane potential depolarization or SOC-activation supports the interpretation that tyrosine kinase activity contributes to the upstream mechanisms of O_2-sensing mechanism in the DA.

Tyrosine phosphatase inhibition mimics O_2-induced DA contraction

Since inhibition of tyrosine phosphorylation inhibited O_2 constriction we next assessed whether increasing phosphorylation would cause DA constriction. Sodium orthovanadate, a non-specific tyrosine phosphatase inhibitor, caused DA contraction and reduced subsequent O_2-induced contraction (fig. 2A–C). When superimposed on oxygen-induced DA contraction, sodium orthovanadate causes little additional vasoconstriction (fig. 2C). The contraction which orthovanadate induced in hypoxic DA rings can be dose-dependently inhibited by the tyrosine kinase inhibitor, genistein (fig. 2D), consistent with genistein's effects on O_2-induced contraction. In a further parallel, DA rings when exposed to 12 hours O_2 and 2 hours hypoxia (recovery) lost most (80%) of the acute constrictor response to oxygen (as reported[31]) and demonstrated a parallel impairment of contraction to sodium orthovanadate (~50%). In contrast, the response to KCl was not affected in this experiment model[31] (data not shown). The parallel pharmacological profiles of orthovanadate, a putative tyrosine phosphatase inhibitor, and O_2 supports the interpretation that tyrosine phosphorylation contributes to O_2-sensing in the DA.

EGFR transactivation is required for O_2-mediated DA contraction

We next attempted to determine the identity of the G protein receptor-coupled tyrosine kinase that is primarily responsible for DA contraction. We compared effects of inhibitors of EGFR versus platelet derived growth factor receptor (PDGFR) on DA tone. The EGFR inhibitor, AG1478 (30 μM) inhibited 80% of the O_2-induced DA contraction (fig. 3A: representative trace; fig. 3D: summary data), whereas pretreatment with the PDGFR inhibitor, AG1296 (30 μM), had no effect (fig. 3D). Moreover, AG1478 was selective for oxygen-induced vasoconstriction and did not significantly reduce KCl vasoconstriction (fig. 3A & supplemental fig. 2). These results indicated that EGFR is the receptor-coupled tyrosine kinase that is primarily responsible for DA contraction and is regulated by the balance between tyrosine kinases and phosphatases.

Because EGFR is known to signal by means of p38 and JAK, we next explored the role of these kinases in DA contraction. AG490 (30 μM), an EGFR and JAK family tyrosine kinase inhibitor, completely inhibited O_2-mediated DA contraction (fig. 3B). EGFR is also known to be phosphorylated and activated by Src[32]. The Src inhibitor PP2 (30 μM) also abolished O_2-mediated DA contraction (fig. 3C). In contrast, the inactive Src control PP3 did not affect O_2-induced DA contraction (fig. 3D). To determine whether the inhibitory effects of blocking EGFR were specific, we also evaluated the effects of a battery of inhibitors of other putative signaling pathways: LFM-A13 (a Bruton's tyrosine kinase inhibitor), GM6001 (a matrix metalloproteinases inhibitor) and concanavalin A (an inhibitor of

endocytotic processes). These did not affect the oxygen-induced DA contraction (data not shown).

p38 and JNK but not the ERK, PI_3 kinase pathways are involved in oxygen-induced DA contraction

To further explore EGFR's downstream signal pathway, we studied the effect of ansomycin (30 μM), an activator of p38 and JNK. Ansomycin pretreatment markedly increased DA tension under hypoxic conditions (increasing tone to approximately 50% of that achieved by O_2-induced contraction, n=5) and reduced subsequent oxygen-induced DA contraction (fig. 4A–B). The inhibition of p38 MAP kinase (SB202190 10 μM) or the inhibition of JNK (JNK inhibitor II, 10 μM) had, as predicted, the opposite effect, significantly attenuating O_2-induced DA contraction (fig 4C). In contrast, neither inhibition of ERK kinase with PD98059 (30 μM), nor inhibition of the PI_3K pathway, with LY294002 (10 μM) or Wortmannin (1 μM), altered O_2-induced DA contraction (fig. 4C). These experiments indicate that p38 and JNK, but not the ERK and PI_3K pathways are involved in O_2-induced DA contraction.

Proper attachment to the extracellular matrix is essential for cell survival[33]. Integrins are transmembrane heterodimeric adhesion receptors that mediate both cell-cell and cell-matrix interactions throughout the body[34]. Cooperation between integrins and EGFR plays an important role in the regulation of cell growth, differentiation, and survival, and it depends on c-Src kinase activities[35]. The integrin receptor ligand gly-arg-gly-asp-ser-pro (GRGDSP) was used to test the possible effect of integrins on O_2-induced DA contraction. Consistent with the proposed role of Src in the activation of EGF-mediated DA contraction, GRGDSP (500 μM) and EGF (1 μg/ml) both caused significant DA contractions (36% or 50% of the maximum O_2-induced contraction, fig. 5A). As observed with oxygen, human EGF, GRGDSP and orthovanadate increased cytosolic calcium in human DASMCs (fig. 5B and 5C). In addition, EGFR siRNA (but not the scrambled control) markedly reduced EGFR mRNA and attenuated O_2-induced increases in cytosolic calcium in DASMC. The O_2-induced fura-2 ratio change was significantly decreased from +0.11±0.01 (scrambled siRNA, n=57) to +0.03±0.0 (siEGFR, n=87); $p<0.01$ (supplemental fig. 3). These data support the concept that Src-mediated activation of EGFR might contribute to O_2-induced DA contraction.

Mitochondria-derived H_2O_2 mediates O_2-induced EGFR phosphorylation in human DASMC

We next studied the acute effect O_2 on EGFR in human DASMC's. Using immunoprecipitation with anti-EGFR, we found that acute oxygen exposure for as little as 3-minutes significantly increased EGFR tyrosine phosphorylation (fig. 6A). By immunostaining technique, we found that EGFR tyrosine residue Y1173 is rapidly phosphorylated by acute (15-minutes) oxygen exposure in human DASMCs (fig. 6B–C).

Previous studies had shown that augmenting intracellular catalase attenuates O_2 sensing in DASMC[36]. We further studied the compartmental specificity of the signaling H_2O_2 effect on EGFR phosphorylation by infecting DASMC with the adenoviral vector containing a mitochondrial-targeted catalase gene (Ad-mitoCAT[6]). O_2-induced EGFR tyrosine Y1173

residue phosphorylation was significantly inhibited by transfection of DASMC with Ad-mitoCAT, versus Ad-empty (each at 100 MOI for 96 hours) (Fig. 6B–C). By qRT-PCR, we also found that EGFR mRNA was also significantly increased in cultured human DASMCs exposed to increased pO_2 for 1 to 72 hours (fig. 6D). These findings are consistent with the interpretation that mitochondrial derived hydrogen peroxide is essential for the oxygen-induced activation (phosphorylation) of EGFR.

Acute O_2 exposure causes EGFR-dependent p38, JNK phosphorylation in human DASMCs

O_2 exposure caused time-dependent phosphorylation of p38 (at thr180/tyr182) and JNK (at thr183/tyr185) in cultured human DASMCs, with a peak response at 3 to 5 minutes (fig. 7A and B). The O_2-induced phosphorylation of p38 and JNK was completely attenuated by the EGFR inhibitor, AG1478 (fig. 7C and D), which suggests that O_2-induced EGFR transactivation is the upstream signal of p38 and JNK phosphorylation (fig. 8).

Discussion

It is widely accepted that receptor tyrosine kinases play an important part in vasoconstriction elicited in response to activation of G protein coupled receptors. In this study we demonstrate that tyrosine kinases play a key role in O_2-induced DA contraction (fig. 1 and fig. 2). Activation of G-protein coupled receptors (GPCRs), in particular EGFR, is involved in DA O_2-sensing. We demonstrate that O_2 causes EGFR transactivation and initiates signaling through a p38/JNK cascade. This signaling pathway requires mitochondrial-derived H_2O_2. The EGFR pathway increases calcium influx and release, resulting in DA contraction (fig. 8).

Mitochondrial-derived H_2O_2-mediated EGFR transactivation is involved in O_2-induced DA contraction

EGF (1 μg/ml) causes about 50% of the maximal O_2 -induced contraction in rabbit DA rings (fig. 5A) and stimulates a robust increase in intracellular calcium (~ 700 nM) in human DASMCs (5B–C). Conversely, the EGFR inhibitor, AG1478 abolished 80% of the O_2-induced DA contraction (fig. 3A and 3D), whereas PDGFR inhibitor, AG1296 had no effect (fig. 3D). The EGFR inhibitor effect on O_2-induced contraction seems specific because it had no significant effect on KCl-induced contraction (fig. 3A, supplemental fig. 2A). These data indicate that EGFR transactivation is required for O_2-mediated DA contraction.

The mitochondrial electron transport chain has been proposed as the DA's O_2 sensor for more than a decade[2; 37; 38]. Inhibitors of either ETC complex I or complex III can fully and selectively abolish and relax O_2-induced DA contraction[2; 38]. Recently we have found that an early step in mitochondrial-based O_2 sensing involves a change in mitochondrial structure. Physiologic increases in O_2 level cause mitochondrial fission. Within 5-minutes of normoxia DASMC mitochondria fragment by means of an actively regulated process (fission). Fission is required for both the pO_2-induced increases in H_2O_2 and vasoconstriction[6]. Inhibition of either mitochondrial fission or mitochondria-derived H_2O_2 production selectively prevents O_2-induced DA contraction without altering contraction to other agonists, such as KCl or phenylephrine[6]. Mitochondrial fission triggers a metabolic

shift in the DASMCs that activates pyruvate dehydrogenase and is required for the observed increases in mitochondrial H_2O_2 production[6]. Our experiments show that EGFR tyrosine residue phosphorylation is dependent on mitochondrial H_2O_2 as shown by mito-catalase inhibition.

Mitochondrial-derived H_2O_2 signals vasoconstriction through at least three known mechanisms: 1). Calcium entry through L-type calcium channels. Roulet et al first reported that O_2 caused membrane depolarization in isolated DA strips[39]. Approximately two thirds of the calcium required for O_2-induced contraction enters the DASMC via the L-type calcium channel[3]. The O_2-sensitive, Kv channels (Kv1.5 and Kv2.1) in human DA are inhibited by O_2 and cause calcium entry through L-type calcium channels[30]. 2). O_2 causes calcium release from the sarcoplasmic reticulum in DASMC and further induces calcium entry through store-operated channels[8; 40]. 3). Rho kinase-related calcium sensitization change, which permits prolonged interaction of actin and myosin at any given Ca^{2+} level[8; 10; 40]. DA contraction ultimately reflects the aggregate effect of increased cytosolic calcium in the cytosol and calcium sensitization.

EGFR regulates the intracellular effects of ligands such as EGF and transforming growth factor-α (TGFα). Upon stimulation, EGFR undergoes a transition from an inactive monomeric form to an active homodimer and stimulates its intrinsic intracellular protein-tyrosine kinase activity. Several tyrosine (Y) residues in the C-terminal domain of EGFR. including Y1173, can be autophosphorylated. EGFR has also been reported to be an H_2O_2-sensitive target[41]. The mechanism involves a direct protein sulfenylation modification of EGFR by H_2O_2 at a critical active site cysteine (Cys797), which enhances its tyrosine kinase activity[41]. In our study, the EGFR tyrosine residue Y1173, was rapidly phosphorylated by acute (3–15 minutes) O_2 exposure (fig. 6A–B). By using mitochondria-specific overexpression of catalase with Ad-mitoCAT in cultured DASMCs, we proved that the mitochondria-specific H_2O_2 is a very important initiator of O_2- induced EGFR phosphorylation (fig. 6B). The significant mRNA up-regulation of EGFR after 1 hour O_2 exposure in cultured human DASMCs (fig. 6D) suggests that EGFR is O_2-sensitive at the transcriptional as well as the post translational level. To confirm molecular specificity, we supplemented the findings using pharmacologic inhibitors of EGFR with siRNA targeting of EGFR in cultured human DASMC. The inhibitory effect of siRNA EGFR on the calcium increases caused by oxygen suggests that EGFR plays an important role on O_2-sensing (fig. 5E). Thus EGFR is sensitive to acute oxygen change and can be quickly transactivated by mitochondria-specific H_2O_2.

EGFR transactivation can be induced by many stimuli including reactive oxygen species and occurs through several pathways[22; 42; 43; 44]. Tseng et al reported that NADPH oxidase-produced superoxide mediates EGFR transactivation by c-Src in arsenic trioxide-stimulated human keratinocytes[45]. Zhuang et al reported that that H_2O_2-induced EGFR transactivation is dependent on the activation of Src, whereas chelation of intracellular Ca^{2+} or inhibition of conventional and novel PKC had no effect on H_2O_2-induced EGFR transactivation in renal proximal tubular cells[21]. They also found that H_2O_2 induced the phosphorylation of Src tyrosine 416, that is required for Src activity, and the phosphorylation of EGFR tyrosine 845, the Src-mediated phosphorylation site. Furthermore, the inhibition of EGFR did not

interfere with H_2O_2-induced Src activation. These data support the concept that Src acts upstream of the EGFR in H_2O_2-treated cells[21]. Our data that Src inhibitor PP2 significantly inhibited O_2-induced DA contraction, whereas its inactive control PP3 had no effect, is consistent with this concept (fig. 3C–D).

Both PTKs and PTPs are sensitive to reactive oxygen species, redox status and oxygen level changes[46; 47; 48]. Reversible oxidation of the active site cysteine residue has recently been identified as a general mechanism for regulation of PTPs. Transient oxidation of thiols in PTPs leads to their inactivation by the formation of either an intermolecular S-S bridge or a sulfenyl-amide bond. Conversely, oxidation of PTKs leads to their activation; either by direct SH modification or, indirectly, by concomitant inhibition of PTPs that leads to sustained phosphorylation of PTKs[49; 50; 51]. By using a modified cysteinyl-labeling assay, we found that PTP activity is decreased after 5–10 min O_2 exposure in human DASMCs (supplemental fig. 4). This may contribute to the overall increase in activity of tyrosine kinases, including EGFR, p38 and JNK (fig. 6A–C; Fig. 7).

p38 MAPK and JNK are EGFR transactivation downstream targets

The MAPK family is the main downstream target of EGFR transactivation[52; 53]. The family includes three subfamilies with multiple members: the extracellular signal-regulated kinases (ERKs), the c-Jun N-amino-terminal/stress-activated protein kinases (JNKs/SAPKs), and the p38 MAPKs. ERK1/2 is typically stimulated by growth factors acting via a tyrosine kinase receptor and is involved in regulation of cell proliferation, whereas p38 and JNK are preferentially activated by cellular stress and are implicated in the regulation of survival, apoptosis, differentiation and contraction, all processes which are important in DA contraction and closure. Sah et al. found that stimulation with EGF causes a biphasic activation of the ERK1/2 MAPK in human ectocervical epithelial cells. The first peak of activation is present at 20 minutes, and the second is present at 36 hours[53]. Kim et al also reported EGF-induced contraction mediated by the activation of ERK1/2, and regulated by the PI3K pathway, in aortic smooth muscle of DOCA-salt hypertensive rats[52].

We found that p38 and JNK specific inhibitors, but not ERK1/2 inhibitor can significantly inhibit O_2-induced DA contraction (fig. 4C). Activation of p38 and JNK with ansomycin increased the hypoxic DA tone and subsequently diminished further O_2- induced DA contraction (fig. 4A–B). Our Western blot data showed that brief O_2 exposure (within 5 minutes) caused p38 and JNK tyrosine residue phosphorylation in human DASMCs (fig. 7A and B). The oxygen-induced phosphorylation of p38 and JNK were completely inhibited by pretreatment with the EGFR inhibitor, AG1478 (fig. 7C and D). These observations suggest that p38 and JNK pathway activation, but not ERK pathway activation, are involved in O_2-induced DA contraction.

p38 MAPK and JNK are involved in HSP27 and caldesmon activation

A classic vasoconstriction pathway is one in which signals activate F-actin activation and cause rearrangement of the cytoskeleton. HSP-27 and caldesmon are two major modulators involved. Activation of p38 MAPK leads to phosphorylation of HSP27 in many cell types including smooth muscle cells. The functions of HSP27 in smooth muscle are not fully

defined, but there is evidence that HSP27 acts as a chaperone or binding partner for Rho and PKC; binds to and modulates interaction of actin, myosin, and tropomyosin; and regulates actin filament structure. Hsp27 and caldesmon are widely reported to be involved in cytoskeleton rearrangement and to contribute to calcium sensitization[54]. The latter plays a role in DA contraction[8,9,10]. However, there is no reported suggestion that they may be involved in oxygen-induced DA contraction. We detected phosphorylation of HSP27 (at the serine 82 residue) and caldesmon (at tyrosine 27 residues) after 3–5 minutes of normoxia in cultured human DASMCs (supplemental fig. 5A and 5B). Thus it appears that both HSP27 and caldesmon are activated by the EGFR/p38/JNK pathway.

Clinical Relevance

The merits of ligation versus the use of drugs, such as indomethacin or ibuprofen, to close the PDA in neonates are still debated[55; 56]. However, it is clear that both approaches have significant side-effects. Consequently, it would be helpful if a better understanding of the cell signaling that controls the normal closure of the DA leads to improved pharmacological intervention. Similarly, although prostaglandin E_1 has been used to maintain DA patency in infants with congenital heart disease, such as pulmonary atresia, for over 35 years [57; 58], it too has side-effects[59]. Again, better understanding of the physiology may improve treatment.

Conclusions

Oxygen-induced DA contraction is mediated in part by EGFR signal transactivation. This transactivation results in p38 and JNK activation and triggers calcium influx resulting in DA contraction. This finding may offer a new opportunity for the development of improved therapies to close the abnormally patent DA in the neonate or to maintain patency in the presence of critical congenital heart disease to provide pulmonary or systemic blood flow.

Limitation

Although our study suggested that EGFR is likely transactivated by the mitochondrial-derived H_2O_2, it remains possible that additional G-protein coupled receptors are also involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key messages

- Oxygen activates epidermal growth factor receptor (EGFR) in ductus arteriosus (DA) smooth muscle cells.
- EGFR inhibition selectively attenuates O_2-induced DA constriction.
- pO_2-induced EGFR activation is mediated by mitochondrial-derived hydrogen peroxide.
- p38 MAPK and JNK mediated EGFR's effects on oxygen-induced DA contraction.
- Tyrosine kinases and phosphatases participate in oxygen-sensing in the DA.
- The EGFR pathway offers new therapeutic targets to modulate patency of the ductus arteriosus.

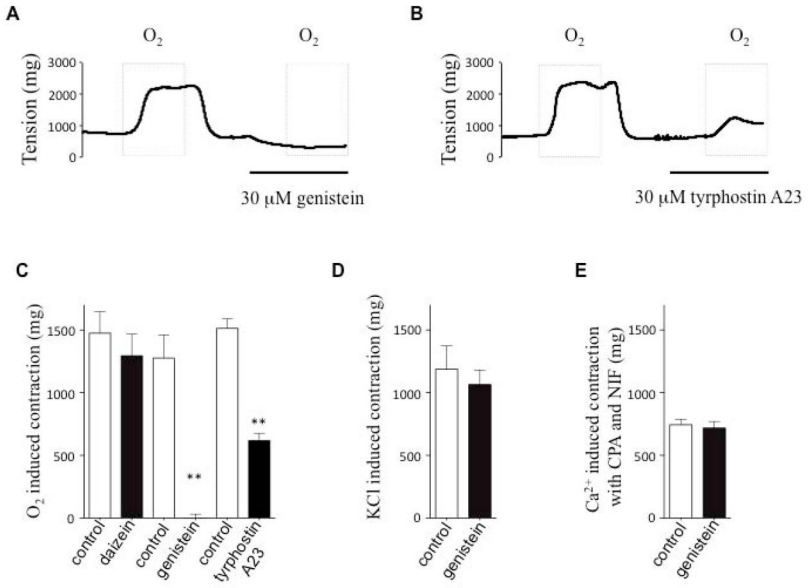

Fig 1. Tyrosine kinase inhibitors reduce the oxygen-induced DA contraction

Different tyrosine kinase inhibitors (genistein and tyrphostin) inhibited oxygen-induced DA contraction (A and B representative traces; C: Summary data. Daizein is negative control for genistein; n=4–7, ** P<0.01. D: Genistein had no effect on KCl-induced contraction, n=6. E: Genistein had no effect on calcium switch from 0 mM to 2 mM induced contraction in the presence of CPA and nifedipine, n=5.

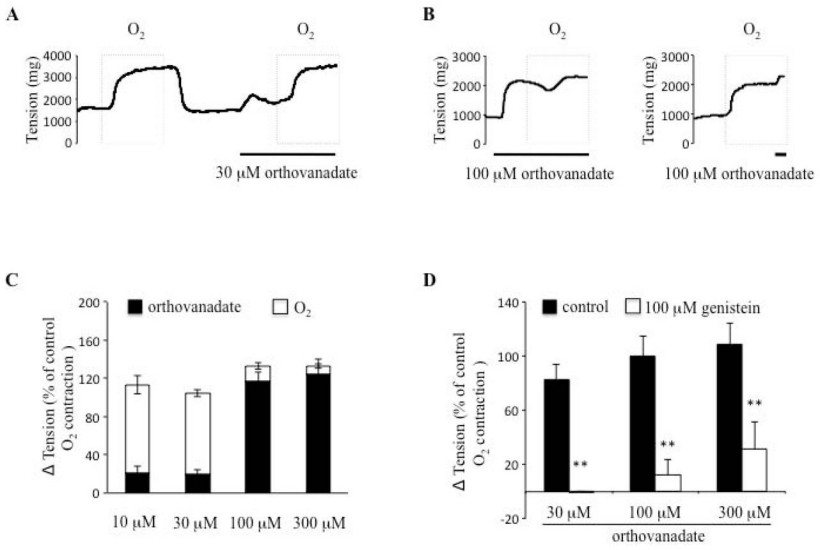

Fig. 2. Orthovanadate, a protein tyrosine phosphatase inhibitor, causes dose-dependent DA contraction in hypoxia

Tyrosine phosphorylation causes DA contraction, dependent on oxygen condition. A:30 μM orthovanadate causes DA contraction in hypoxia. B: Orthovanadate causes dose-dependent DA contraction and subsequently prevents "additional" O_2-induced DA contraction. C: Orthovanadate dose-response contraction, (n=3–5). D: Genistein reduces orthovanadate-induced DA contraction in hypoxia (n=5).

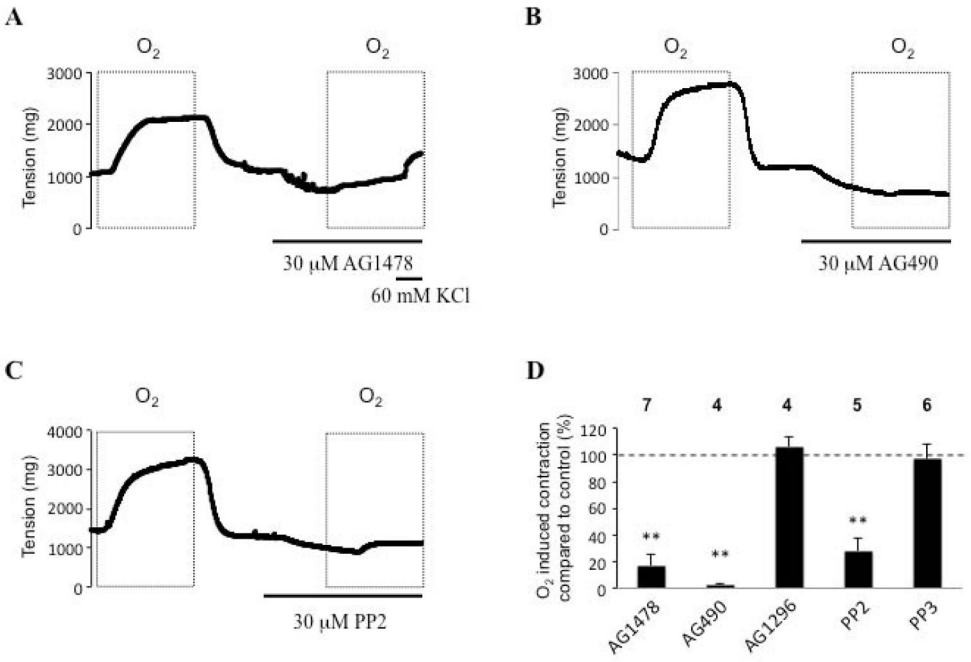

Fig 3. EGFR inhibitor abolished O_2-induced DA contraction

Effects of (A): AG1478 (EGFR inhibitor), (30 μM AG1478 had no effect on KCl-induced contraction), (B): AG490 (EGFR and Jak family tyrosine kinase inhibitor) and (C): PP2 (Src family protein tyrosine kinases inhibitor) on oxygen-induced DA contraction. (D): summary data and n number. Note: PDGF inhibitor (AG1296) had no effect on O_2-induced contraction. AICAR is an activator of AMP kinase.

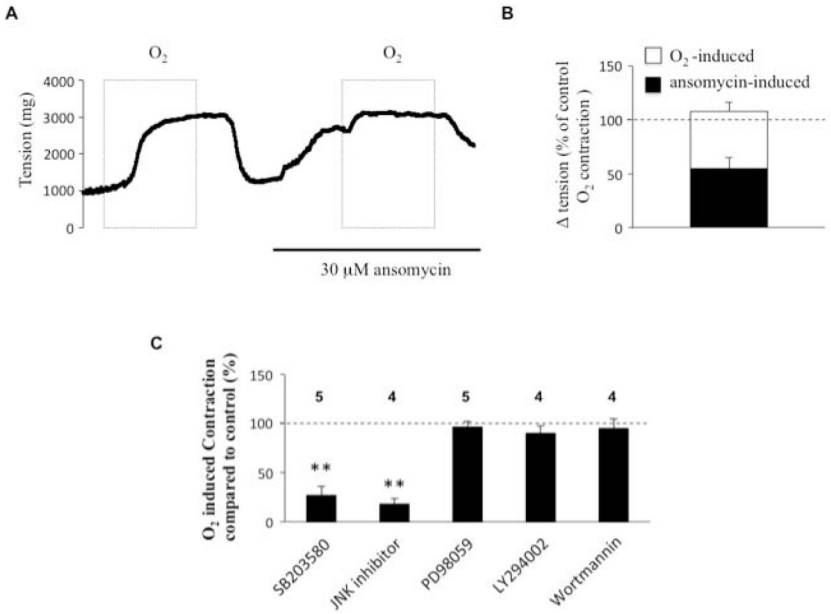

Fig 4. p38 and JNK but not the ERK pathway are involved in O_2-induced DA contraction

(A) and (B): Relationship of the effect of ansomycin (p38 and JNK activator) on DA tension change and subsequent O_2-induced DA tension change (n=5). (C) Different inhibitor experiments indicate that the p38 and JNK pathways are necessary for O2-mediated DA contraction. SB203580 (p38 inhibitor); PD98059 (ERK inhibitor); LY294002 and Wortmannin (PI3-kinase inhibitor).

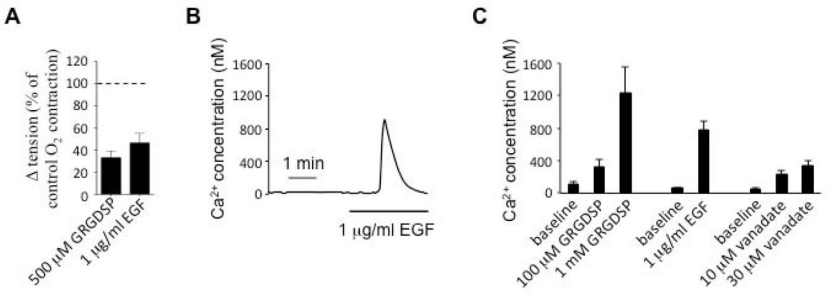

Fig 5. EGF causes DA tension change and calcium influx

A: Integrin ligands GRGDSP and EGF cause strong DA contraction (n=4–6). B: As observed with oxygen, EGF causes calcium influx and increases cytosolic calcium in human DASMCs. C: Calcium image summary data for integrin ligands GRGDSP, EGF and vanadate (n=15–36).

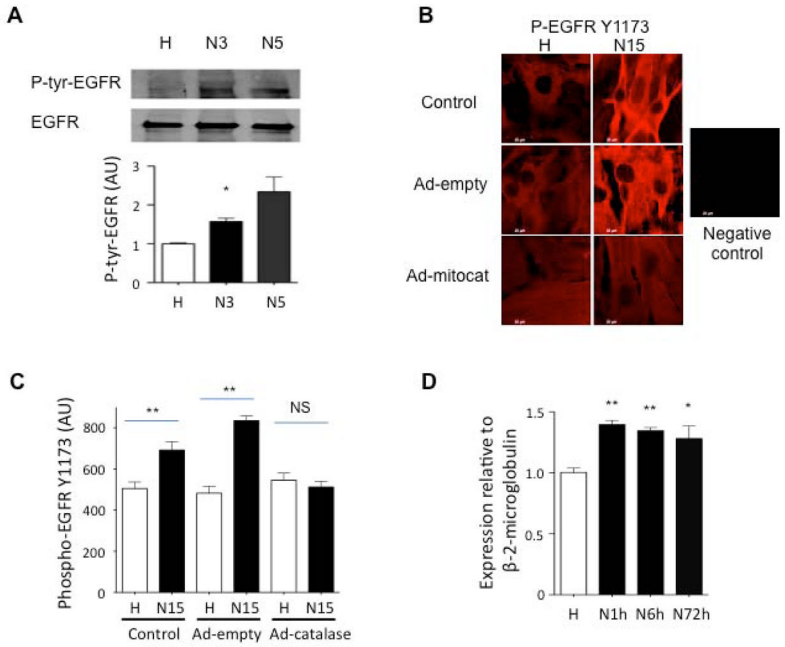

Fig 6. Acute O_2-induced EGFR phosphorylation in human DASMCs

A: Consistent with the importance of EGFR in O_2-contraction, a crucial EGFR tyrosine residue was phosphorylated in 3 human DA smooth muscle cell lines after 3 to 5 minute normoxia. * P<0.05, n=5, Vs hypoxia control by immunoprecipitation technique with anti-EGFR and anti-tyrosine. B: EGFR tyrosine residue Y1173 was phosphorylated by 15 minutes O2 exposure by immunostaining. Mitochondria-specific overexpression of catalase with Ad-mitoCAT abolished the effect. C: summary data for immunostaining (n=36–50). D. EGFR mRNA was significantly increased after 1 to 72 hours normoxia in cultured human DASMCs by qRt-PCR. (* P<0.05, Vs hypoxia control. * P<0.05, ** P<0.01 Vs hypoxia control, n=4).

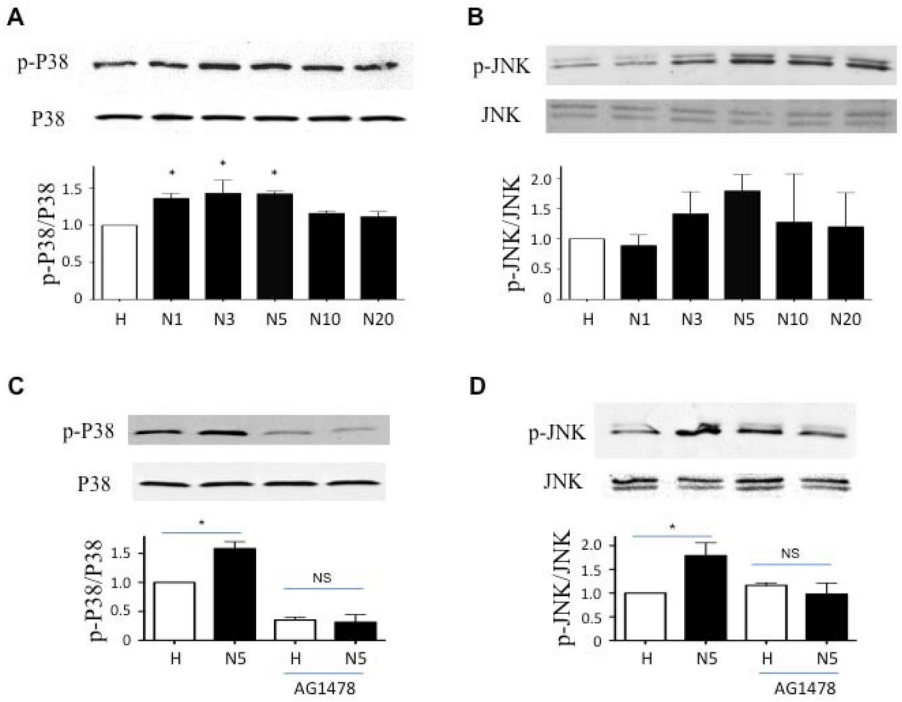

Fig 7. The O_2-induced phosphorylation of p38 and JNK is completely reduced by the EGFR inhibitor, AG1478

p38 and JNK were phosphorylated in cultured human DASMCs within 5 minutes of normoxia. (A and B) and can be significantly inhibited by EGFR inhibitor, AG1478 (C and D) (n=3–6).

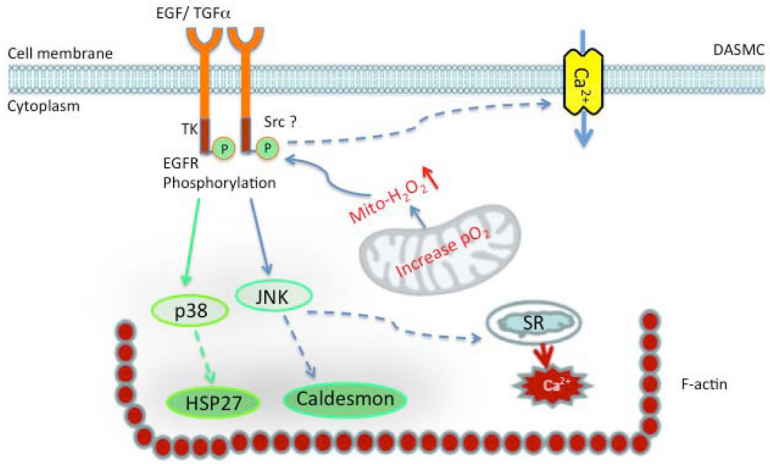

Fig. 8.

O_2 induces EGFR signal pathway transactivation in DASMCs. O_2 generates mitochondrial H_2O_2 and results in tyrosine phosphorylation of EGFR. Subsequently, the EGFR signal pathway activates p38 and JNK. This results in phosphorylation of HSP27 and caldesmon and Ca^{2+} release. Ultimately EGFR activation alters F-actin structural arrangement. The dotted lines indicate possible mechanisms.