CALL FOR PAPERS | Cardiovascular Mitochondria and Redox Control in Health and Disease

20-HETE-induced mitochondrial superoxide production and inflammatory phenotype in vascular smooth muscle is prevented by glucose-6-phosphate dehydrogenase inhibition

Anand Lakhkar,1 Vidhi Dhagia,1 Sachindra Raj Joshi,1 Katherine Gotlinger,1 Dhara Patel,2 Dong Sun,2 Michael S. Wolin,2,3 Michal L. Schwartzman,1 and Sachin A. Gupte1,3

1Department of Pharmacology, New York Medical College School of Medicine, Valhalla, New York; 2Department of Physiology, New York Medical College School of Medicine, Valhalla, New York; and 3Translational Centre for Pulmonary Hypertension, New York Medical College School of Medicine, Valhalla, New York

Submitted 15 December 2015; accepted in final form 23 February 2016


20-Hydroxyeicosatetraeonic acid (20-HETE), the ω-hydroxylation metabolite of arachidonic acid (AA), is produced by cytochrome P-450 monoxygenases of the (CYP4A and CYP4F gene) families in an NADPH-dependent manner (49). 20-HETE is proinflammatory and it regulates vascular and renal function (49). It also plays a critical role in the pathogenesis of systemic hypertension, renal stenosis, and atherosclerosis (27, 63, 65). 20-HETE increased by hypoxia inhibits hypoxia-induced pulmonary artery constriction (69). However, its role in the hypoxia-induced inflammation of pulmonary arteries or lungs is yet unclear.

Recently, our laboratory also found that 20-HETE is involved in promoting prolonged hypoxia-induced pulmonary vasoconstriction and that glucose-6-phosphate dehydrogenase (G6PD), which is a major producer of NADPH in the cell, and cytochrome P-450 monoxygenase enzymes are functionally coupled in vascular smooth muscle tissue (unpublished observations). G6PD inhibition relaxes pulmonary arteries by decreasing intracellular calcium (20), prevents switching of vascular smooth muscle cells to a synthetic phenotype (9, 10), and reduces pulmonary hypertension (9, 47). However, whether prolonged hypoxia-induced 20-HETE-synthesis is G6PD dependent or independent and whether G6PD modulates 20-HETE signaling in the vascular smooth muscle remain unknown. Various cell types in the lungs produce 20-HETE. Notably, airway and peripheral lung tissues produce the most 20-HETE (70). Since 20-HETE is an autacoid, we studied the effects of G6PD inhibition on extracellular 20-HETE-elicited cell signaling. Proving the existence of such an interaction will be of clinical importance.

Increased production of 20-HETE in vascular tissue or application of 20-HETE to blood vessels stimulates vascular smooth muscle cell contraction (52) as well as reactive oxygen species production (37, 53). 20-HETE activates mitogen-activated protein kinase (MAPK) 1 and 3 (also known as extracellular signal regulated kinase 2 and 1, respectively) and promotes secretion of cytokines, including TNF-α, IL-8, and...
IL-6 (6, 30, 40, 59). However, the sources of reactive oxygen species stimulated by 20-HETE in vascular smooth muscle cells remain unknown and the mechanisms through which 20-HETE stimulate the secretory or synthetic phenotype of vascular smooth muscle cells are not clearly understood. Therefore, this study was undertaken to test the hypothesis whether the inhibition of G6PD prevents 20-HETE production and 20-HETE-induced proinflammatory signaling that promotes secretory phenotype of vascular smooth muscle cells.

MATERIALS AND METHODS

All physiological buffers [Krebs, 120 mM KCl (HK), 30 mM KCl (30K), and HEPEs] were prepared by using salts (analyzed reagent grade) from Baker Chemical. All gas tanks were purchased from Air Gas (Allentown, PA). Dehydroepiandrosterone (DHEA), 6-amino-nicotinamide (6-AN), and maleimide were obtained from Sigma Chemical (St. Louis, MO). 20-HETE was purchased from Cayman Chemical (Ann Arbor, MI) and 6-amino-nicotinamide (6-AN) was purchased from Tocris. All experiments were performed following the New York Medical College Animal Care and Use Committee-approved protocol in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult male G6PD mutant mice and appropriate age-matched wild-type (WT) controls were used. The mutation results in decreased translation of G6PD and leads to ~20–40% residual G6PD activity G6PD mutant mice compared with the WT littermate control mice (26, 48).

Tissue preparations. Bovine lungs were obtained from the local slaughterhouse in ice-cold physiological-buffered saline (PBS). The third order branches of pulmonary artery were used for experiments as published previously (25). Pulmonary arteries were cleaned of their connective tissue and cut into rings of 2- to 3-mm diameter and width. Thoracic aorta was isolated from male Sprague-Dawley rats (340 g) and 2- to 3-mm length rings were used as described in previous studies (23). Endothelium was removed by rubbing the lumen with wooden stick. Freshly prepared blood vessel rings were used in studies for vascular reactivity and Western blot protein analysis.

Measurement of vascular reactivity in bovine pulmonary arteries. Endothelium-denuded artery rings were mounted on Radnoti Instruments’ force displacement transducers for recording isometric force development through Powerlab data acquisition system from AD Instruments, as previously described (20). Arterial rings were incubated in Krebs-bicarbonate buffer containing 118 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl₂, 25 mM NaHCO₃, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, and 5.6 mM glucose for 1 h under resting tension of 5 g. In RESULTS, interventions were conducted under prolonged hypoxia (40 mM H₂O₂ and 20-HETE-induced proinflammatory signaling that promote secretory phenotype of vascular smooth muscle cells.

Western blot analysis. Freshly prepared arterial rings were flash frozen by using liquid nitrogen. These frozen arterial rings were pulverized and homogenized in NP-40 lysis buffer (50 mMol/l Tris-HCl pH 7.4, 150 mMol/l NaCl, and 0.5% NP-40) containing protease and phosphatase inhibitors. The expression level of all the proteins except protein kinase G1α (PKG1α) was obtained under reducing condition as described previously (36). Thiol reducing conditions were avoided in the samples analyzed for PKG1α dimer (by not using β-mercaptoethanol). Maleimide (100 mM) was added in the lysis buffer in samples where PKG was measured to alkylate the thiols with Tween-20 for 1 h. Subsequently, membranes were incubated in primary and secondary antibodies as per manufacturer’s protocol. Protein bands were visualized with an enhanced chemiluminescence kit (Pierce 32106) on X-OMAT autoradiography film (Kodak). Protein levels were measured using densitometry analysis by the ImageJ software. The following antibodies were used for immunoblotting: total Elk (A-303-530 A; Bethyl Laboratories), PKG1α (SC10338; Santa Cruz Biotechnology), p-ELK-1 (SC8406; Santa Cruz Biotechnology), TNF-α (SC-292640; Santa Cruz Biotechnology), pNFATc3 (SC-8405; Santa Cruz Biotechnology), tNFATc3 (SC-365786; Santa Cruz Biotechnology), β-actin (A5441; Sigma), pErk1/2 (p42/44; 9101; Cell Signaling), Erk1/2 MAP kinase (p24/24; 4695; Cell Signaling), total VASP (3132; Cell Signaling), and phospho VASP (3114; Cell Signaling).

Superoxide measurement using chemiluminescence. As published previously by our laboratory (24), changes in superoxide were measured from quantifying the chemiluminescence of 5 μM lucigenin in a liquid scintillation counter (LS6000IC; Beckman Instruments, San Diego, CA) with a single active photomultiplier tube in a dark room. Initial background chemiluminescence (blank readings) was measured in plastic scintillation minivials containing only 5 μM lucigenin in 1 ml of Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4) in the absence of tissue. Right after obtaining the blank readings, arterial rings were added into each vial to measure the chemiluminescence in presence of the tissue (tissue readings). Blank measurement was subtracted from subsequent measurements made in the presence of arterial rings. The rings were weighed at the end of the experiment. The counts (tissue-blank) were divided by weight to gain the final data in counts per minute per gram of tissue.

Superoxide measurement using HPLC. Measurement of the superoxide-specific hydroxylated products of MitoSox and dihydroethidium were employed for quantifying changes in mitochondrial and extra-mitochondrial superoxide, using previously described methods (71). Before the start of the experiment increasing concentrations of Mito-2-hydroxyethidium or 2-hydroxyethidium were loaded into the column to generate a standard curve. Third order bovine pulmonary arteries were incubated with 20-HETE (1 μM) in the presence and absence of G6PD inhibitors, DHEA (300 μM), and 6-AN (3 mM) for 12 h and were then treated with either 5 μM MitoSox or dihydroethidium (DHE) for 1 h in the dark to measure mitochondrial and extra-mitochondrial superoxide, respectively. They were washed several times with Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4) and then flash frozen with liquid nitrogen. Tissues were first weighed and then pulverized in the presence of liquid nitrogen, dissolved in a solution of 100% acetonitrile (HPLC grade). These samples were incubated at −20°C for 1 h. After 1 h, samples were centrifuged and the supernatant was used for HPLC analysis of the superoxide-specific hydroxylated product of MitoSox (Mito-2-hydroxyethidium) or of dihydroethidine (2-hydroxyethidium) using an HPLC system with a Jasco FP-1520 fluorescence detector and a Beckman ultrasphere reverse column (C18, 5 μ, 250 × 4.6 mm).

Liquid chromatography-tandem mass spectrometry: analysis of 20-HETE and PGE₂. For the mass spectrometry analysis we used endothelium denuded third order bovine pulmonary arteries and lungs from G6PD mutant and WT mice. The arterial rings were incubated in tissue baths for 12 h under hypoxia or aerobic conditions after giving them passive tension of 5 g and were either treated with DHEA (300 μM) or 6-AN (3 mM) or were left untreated. After 12 h the arterial...
G6PD REGULATES 20-HETE SIGNALING

RESULTS

G6PD inhibition or knockdown decreased endogenous production of 20-HETE. Synthesis of 20-HETE by CYP4A and 4F is dependent on NADPH (5). In the vascular smooth muscle cells a majority of NADPH is produced by G6PD and 6PGD. Therefore, we measured 20-HETE levels in the aerobic and prolonged hypoxic pulmonary arteries treated without and with G6PD inhibitors. 20-HETE was undetectable in pulmonary arteries under aerobic conditions, and its levels increased (3.1 ± 0.6 pg/mg tissue) within 30 min after the vessels were exposed to hypoxia, and it remained elevated for 12 h (Fig. 1A). G6PD inhibition reduced (P < 0.05; Fig. 1A) 20-HETE and PGE2 levels were not affected by G6PD inhibition (control: 16.75, DHEA: 17.88, and 6-AN: 17.89 pg/mg). We also found that 20-HETE levels were significantly less in the lungs of G6PD mutant compared with WT mice (Fig. 2B). CYP activity was not modified by 6-AN (3 mM; 61 ± 3 pg 20-HETE/h/25 μg microsomes; n = 5) vs. control (76 ± 3 pg 20-HETE/h/25 μg microsomes; n = 5).

G6PD inhibition increased PKG activity without increasing intracellular cGMP and decreased 20-HETE production in a PKG-dependent manner. Previous work done in our laboratory has demonstrated that G6PD activation contributes to the development of hypoxic pulmonary vasoconstriction (20) and inhibition of G6PD with 6-AN (1 mM) blocked it in a PKG-independent and -dependent manner (10, 21). To determine whether G6PD inhibition activated PKG, we treated the arterial rings with DHEA and 6-AN for 12 h. G6PD inhibition by DHEA (100 μM) and 6-AN (1 mM) did not increase cGMP (Fig. 2A) but concurrently increased PKG1α dimer (Fig. 2C), the physiologically active form of the kinase (17, 34, 35), and augmented phosphorylation of VASP at Ser239 (Fig. 2B), which is an indicator of PKG activity, in the same samples. It is noteworthy that a competitive and irreversible inhibitor of PKG, 2-bromo-3,4-dihydro-3-[3,5-O-[4R]-mercaptoprophosphinylidene]-β-d-ribofuranosyl]-6-phenyl-9H-imidazo[1,2-a]purin-9-one sodium salt (Rp-cGMPs; 100 nM), decreased DHEA-mediated PKG1α dimer expression (Fig. 2C).

Next, we determined whether PKG signaling-mediated G6PD inhibition induces reduction of 20-HETE levels in pulmonary arteries. We incubated the arteries in the absence or presence of the PKG inhibitor Rp-cGMPs (100 nM) and the G6PD inhibitor DHEA. Interestingly, inhibition of PKG with Rp-cGMPs partially reversed the suppression of 20-HETE by DHEA-mediated PKG1α dimer expression (Fig. 2C).
G6PD inhibition prevented 20-HETE-mediated downregulation of PKG1α dimer expression and upregulation of pErk1/2. To determine the signaling pathways through which 20-HETE evokes inflammation, we measured the PKG1α dimer-to-monomer ratio and pErk1/2. Application of 20-HETE (1 µM) to pulmonary arteries decreased PKG1α dimer-to-monomer ratio and increased pErk1/2 (Fig. 3, A and B). G6PD inhibition blocked the 20-HETE-induced decrease in the PKG1α dimer-to-monomer ratio and the increase in pErk1/2.

G6PD inhibitors via activation of PKG blocked 20-HETE-induced production of mitochondrial superoxide in the pulmonary arteries. 20-HETE stimulates reactive oxygen species that are proinflammatory, -migratory, and -proliferative (49, 64, 66). Previous studies reported that 20-HETE increases superoxide production (detected by DHE fluorescence by microscopy) in aortic and pulmonary artery endothelial cells (7, 37). Since this approach to detect superoxide is semiquantitative and is somewhat nonspecific, we employed HPLC methods to determine extra-mitochondrial and mitochondrial superoxide levels in pulmonary arteries in response to 20-HETE. Mitochondrial but not extra-mitochondrial derived superoxide production was stimulated by 20-HETE (1 µM) under aerobic conditions (Fig. 4, A and B) and hypoxia (6585.385 nmol/g of tissue). In contrast, dibromo-dodecenyl-methylsulfimide (DDMS), a potent inhibitor of CYP4A (1 µM), blocked mitochondrial superoxide production in the pulmonary arteries under chronic hypoxia (Fig. 4C).

Since G6PD-derived NADPH regulates superoxide production from NADPH oxidases (22), we examined whether 20-HETE-elicitates generation of superoxide in a G6PD-dependent manner. DHEA and 6-AN blocked the increase in 20-HETE-elicited superoxide production. Next, we investigated whether 20-HETE-induced superoxide production was reduced by G6PD inhibitor(s) in a PKG-dependent manner. Therefore, we treated pulmonary arteries with 20-HETE for 12 h in tissue baths after pretreating them with either DHEA or 6-AN alone or in the presence of Rp-cGMPs and then measured superoxide production by lucigenin chemiluminescence method. Inhibition of 20-HETE-induced superoxide productions by DHEA and 6-AN was partly reversed by Rp-cGMPs treatment (Fig. 4D). Additionally, we found that 2-aminoethoxydiphenyl borate (2APB; 60 µM), an antagonist of the inositol 1,4,5-triphosphate (IP3) receptor, blocked the increase in mitochondrial superoxide elicited by 20-HETE [in arbitrary units/g tissue: control (221,998 ± 32,463); 20-HETE (565,749 ± 123,822); and 2APB + 20-HETE (114,705 ± 42,092)].

Fig. 2. Protein kinase G1α (PKG1α), phosphorylated-VASP, and cGMP levels in bovine pulmonary arteries untreated and treated with G6PD inhibitors. A and B: expression of PKG1α measured as dimer-to-monomer ratios and phosphorylated-VASP increased by inhibiting G6PD with DHEA (100 µM) or 6-AN (1 mM). C: cGMP levels in the samples are not changed. n = 5 in each group. *P < 0.05 vs. control; #P < 0.05 vs. treatments.
20-HETE-induced expression of TNF-α by pulmonary arteries is blocked by G6PD inhibitor(s). Inflammation is an important contributor to the pathogenesis of systemic and pulmonary hypertension (39). Since proinflammatory cytokine TNF-α is involved in the acute phase reaction and IL-6 evokes late phase reaction, we determined TNF-α and IL-6 expression in the arteries/pulmonary artery smooth muscle cells exposed to 20-HETE. TNF-α has a soluble form and a transmembrane form (28), both of which are involved in the inflammatory response. We were able to detect the transmembrane form in the pulmonary artery smooth muscle. TNF-α expression increased (P < 0.05) in arteries (Fig. 5A) and smooth muscle cells (data not shown) exposed to 20-HETE. 20-HETE increased IL-6 level (in pg/ml; control: 266 ± 44 and 20-HETE: 443 ± 97; P < 0.05).

Next, we investigated if inhibition of G6PD decreased 20-HETE-induced expression of TNF-α and if this is mediated via PKG. We treated pulmonary arteries with 20-HETE for 12 h after pretreating them with either DHEA or 6-AN alone or in the presence of Rp-cGMPs. DHEA and 6-AN decreased 20-
HETE-induced expression of TNF-α in a PKG-dependent manner (Fig. 5A).

20-HETE increased Elk-1 expression and pretreatment of arteries with G6PD inhibitor(s) decreased Elk-1 expression. Elk-1 drives transcription of Tnfα gene (19). Transcriptional activation activity of Elk-1 is increased by Erk1/2 (MAPK)-dependent phosphorylation at Ser383 and conversely is decreased by PKG1α-dependent sumoylation (11, 31). Since 20-HETE decreased PKG1α and increased pErk1/2, we estimated Elk-1 expression status in arteries treated with the CYP4A inhibitor DDMS and in arteries treated with 20-HETE in absence and presence of the G6PD inhibitors DHEA or MitoTempol. DDMS decreased Elk-1 expression in pulmonary arteries under prolonged hypoxia (Fig. 5B). 20-HETE upregulated Elk-1 expression (Fig. 5C), which was decreased by the pretreatment with DHEA and by MitoTempol (1 μM).

20-HETE decreased miR-143 and miR-133a in rat aorta and increased Elk-1 expression in aortic smooth muscle cells. It is established that miR-145/143 and miR-1/133a are involved in the regulation of smooth muscle cell phenotype (32). miR-143 suppresses the expression of Elk-1 (12, 46). Activation of Erk1/2 (MAPK1/3) downregulates miR-133a expression, and this promotes a synthetic phenotype in vascular smooth muscle cells in vitro and in vivo leading to remodeling of blood vessels (57). Therefore, we asked the question whether 20-HETE affects the expression of miR-133a and miR-143? We used rat aorta and aortic smooth muscle cells to study the effect of 20-HETE on miR expression and concomitantly on the miR-143 target, Elk-1. We applied 20-HETE (0.1 and 1 μM) to isolated rat aorta for 12 h and then measured miR-1/133a and miR-145/143 by QPCR. 20-HETE did not significantly affect the expression of miR-1 or miR-145 (Fig. 6, A and C). Expression of miR-133a (Fig. 6B) and miR-143 (Fig. 6D) in aorta was decreased by application of 20-HETE in a dose-dependent manner. Since 20-HETE elevated reactive oxygen species and G6PD inhibitors decreased it, we also examined if H2O2 and G6PD inhibitors regulated miR-143 levels. We found that H2O2 decreased and G6PD inhibitors increased miR-143 levels in aortic smooth muscle cell line (A7r5; Fig. 6E). Furthermore, we observed that G6PD inhibition by DHEA and mitochondrial ROS by MitoTempol decreased the 20-HETE-induced increase in Elk-1 in A7r5 cells (Fig. 6, F and G).

**DISCUSSION**

The detection of G6PD and CYPs complex in the vascular smooth muscle identified by proteomic analysis (Table 1), along with the observation that G6PD inhibition or knockdown decreased 20-HETE levels, suggests G6PD and CYP4-ω-hy-

**Fig. 5.** 20-HETE increased TNF-α and Elk-1 expression and this was blocked when G6PD was inhibited. A: incubation of bovine pulmonary arteries with 20-HETE (1 μM) significantly increased the expression of TNF-α. The increase in expression of TNF-α was blocked when the vessels were pretreated with the G6PD inhibitors DHEA and 6-AN. *P < 0.05 vs. control; $P < 0.05 vs. 20-HETE. B: inhibition of endogenous 20-HETE levels by DDMS reduced the expression of ELK-1, which is a transcription factor for TNF-α. *P < 0.05 vs. control. C: 20-HETE increased Elk-1 expression in the endothelium denuded bovine pulmonary arteries. Increase in Elk-1 expression by 20-HETE was inhibited by DHEA and MitoTempol (1 μM). *P < 0.05 vs. control; n = 5 in each group.
Droxylases are functionally coupled in vivo. These findings complemented earlier studies, which showed that CYP4A and 4F family monooxygenases generated 20-HETE in a NADPH-dependent manner (4, 44). Furthermore, 20-HETE is increased by hypoxia in the pulmonary artery and G6PD inhibition blocked the synthesis of 20-HETE. Since G6PD inhibition blocked 20-HETE signaling, it is reasonable to propose that inhibition of 20-HETE-dependent signaling pathways that elicit constriction and inflammation of the arteries are potential mechanisms through which G6PD inhibition decreases hypoxia-induced pulmonary vasoconstriction and pulmonary hypertension (9). Therefore, our findings indicated that G6PD-derived NADPH is a driver for 20-HETE-synthesis and that upregulation of G6PD expression or activity not only increases endogenous levels of 20-HETE but also modulates 20-HETE signaling in vascular diseases such as pulmonary hypertension.

20-HETE is pro-proliferative, -inflammatory, and -migratory (49, 66), all of which contribute to the pathogenesis of systemic and pulmonary hypertension. Although the increase in 20-HETE by hypoxia inhibited acute hypoxia-induced pulmonary artery constriction (2, 69), it time and dose dependently increased superoxide production from NADPH oxidases (42).

Table 1. Proteomic analysis by LC/MS/MS showing a complex of G6PD with various CYP450 enzymes in G6PD pull down from bovine arteries

<table>
<thead>
<tr>
<th>Top Ranked Protein Name</th>
<th>Accession No.</th>
<th>Protein MW</th>
<th>Protein PI</th>
<th>Peptide Count</th>
<th>Total Ion Score</th>
<th>Total Ion CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP450, family 2, subfamily C, Polypeptide 87 precursor (Bos taurus)</td>
<td>gi 115497566</td>
<td>55.895</td>
<td>7.2</td>
<td>2</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>CYP450 2C21 (Bos taurus)</td>
<td>gi 297464426</td>
<td>48.686</td>
<td>7.6</td>
<td>5</td>
<td>84</td>
<td>100</td>
</tr>
<tr>
<td>CYP450 2C19 (Bos taurus)</td>
<td>gi 297464424</td>
<td>48.475</td>
<td>6.3</td>
<td>2</td>
<td>45</td>
<td>99</td>
</tr>
<tr>
<td>CYP450 subfamily 2B (Bos taurus)</td>
<td>gi 296477790</td>
<td>50.607</td>
<td>8.2</td>
<td>2</td>
<td>38</td>
<td>95</td>
</tr>
</tbody>
</table>

LC/MS/MS, liquid chromatography tandem mass spectrometry; G6PD, glucose-6-phosphate dehydrogenase; MW, molecular weight; CI, confidence interval; PI, isoelectric point.
in the cultured pulmonary artery endothelial cells (37). Also, studies have reported that 20-HETE-induced superoxide mediated flow-induced constriction of cerebral arteries (58). Our current results further demonstrated that inhibition of 20-HETE biosynthesis by DDMS decreased mitochondrial superoxide generation and conversely application of 20-HETE to endothelium denuded pulmonary arteries for 12 h elicited superoxide generation from mitochondria but not from extra-mitochondrial sources. This led us to the question of how does 20-HETE increase mitochondrial superoxide generation? One potential explanation was 20-HETE passed through the gap junctions (unpublished data) and stimulated mitochondrial superoxide. Alternatively, since 2APB blocked 20-HETE-induced superoxide (see RESULTS) and 20-HETE stimulates Ca\(^2+\) release from IP\(_3\) receptors potentially through a PLA\(_2\) or G protein-coupled receptor-dependent mechanism in airway smooth muscle cells (50), the data suggest that 20-HETE potentially triggered mitochondrial Ca\(^2+\) overload through SR-mitochondrial coupling, which is well known to increase mitochondrial respiration/metabolic rate and superoxide levels (13, 33). Although the functional role of mitochondrial vs. extra-mitochondrial superoxide in the development of vascular diseases is not yet well characterized, elevated superoxide in the cell has been shown to inactivate nitric oxide (NO) and soluble guanylate cyclase-PKG or activate Erk1/2 signaling pathways (24, 38) and contribute to the pathogenesis of vascular diseases including pulmonary hypertension (18, 36). Therefore, from our current findings it is reasonable to suggest that elevated mitochondrial superoxide mediated, at least partly, 20-HETE-induced pulmonary artery dysfunction.

Superoxide is mitogenic and proinflammatory in vascular smooth muscle cell (56). Along these lines we found that 20-HETE, which increased mitochondrial superoxide, robustly increased expression of proinflammatory cytokine TNF-α and IL-6 in pulmonary artery and pulmonary artery smooth muscle cell, respectively. Interestingly, G6PD inhibition decreased mitochondrial superoxide production and TNF-α expression elicited by 20-HETE. Transcription of the Tnfα gene is elevated by reactive oxygen species-induced NF-κB activation (51) and is also increased by Elk-1 (19). 20-HETE is a known activator of NF-κB (30). Here, we also found that 20-HETE upregulated and DDMS downregulated Elk-1 expression, respectively, in pulmonary artery. Although we did not investigate whether the smooth muscle cells or other types of cells in the arterial wall produced 20-HETE, our findings suggested that signaling pathways stimulated by both endogenous and exogenous 20-HETE regulated Elk-1 expression. Furthermore, 20-HETE applications to the rat aorta decreased miR143, which is known to inhibit Elk-1 expression (12), and suppressed miR-133a, which prevented expression of synthetic (secretory/proinflammatory) phenotype in vascular smooth muscle cells (57). Therefore, these findings suggested that 20-HETE-induced transformation of the vascular smooth muscle cells from contractile to secretory/proinflammatory phenotype that increased Tnfα is mediated through the miR143-Elk-1 pathway and inhibition of G6PD or mitochondrial superoxide generation by MitoTempol prevented this.

The TNF-α-induced increase in cGMP paradoxically downregulates PKG1α expression in the bovine aortic smooth muscle cells (3). Therefore, it is reasonable to speculate that 20-HETE-induced overexpression of TNF-α downregulated PKG1α. Interestingly, G6PD inhibition decreased 20-HETE and rescued 20-HETE-induced downregulation of PKG1α and increase of pErk1/2. Since inhibition of G6PD-derived NADPH redox oxidized Cys42 residues on PKG and activated it (10), we proposed that application of G6PD inhibitors to pulmonary arteries for 12 h oxidized Cys42 residues that either prevented or overruled the 20-HETE-induced decrease of PKG1α leading to an increase in the PKG1α dimer and VASP phosphorylation without increasing intracellular cGMP levels. Previous studies showed that PKG1α dimer is elevated in vascular tissue by DHEA (43, 45). Furthermore, the increase of PKG activity by G6PD inhibition decreased 20-HETE levels as well as 20-HETE-induced mitochondrial superoxide generation. This suggests that either PKG inhibited CYP4 activity or downregulated hypoxia-induced CYP4 expression. In this regards, NO is known to block CYP4-derived 20-HETE (1, 62) and increased NO-mediated activity of cGMP/PKG has been associated with downregulation of CYP4A enzymes in various organs and renal arteries of a LPS-treated rat model of septic shock (60). The increase of PKG activity somoylates Elk-1 and inactivates it (11). In contrast, the increase of MAPK1/Erk2 activity phosphorylates Elk-1 at Ser383 and stimulates its transcriptional activity (68). Accordingly, since G6PD inhibition upregulated miR-143 expression and decreased 20-HETE-induced Elk-1 expression, we proposed that increased PKG and decreased Erk1/2 activity by G6PD inhibition antagonized the 20-HETE-induced increase of Elk-1 leading to reduction of TNF-α expression. Moreover, DHEA and 6-AN decreased mitochondrial superoxide and increased miR-143, H\(_2\)O\(_2\) down-

![Fig. 7. Summary model. 20-HETE is synthesized from arachidonic acid (AA) by CYP4 group of enzymes, which utilize NADPH generated by G6PD. 20-HETE causes an increase in mitochondrial reactive oxygen species (ROS; superoxide (O\(_2\)-) and hydrogen peroxide (H\(_2\)O\(_2\))) and a decrease in miR-143, p42/44 MAP kinase phosphorylation, which then leads to increased expression of ELK-1. Increased expression of ELK-1 then leads to an increase in expression of TNF-α, which can then block PKG signaling in the vascular smooth muscle. Inhibition of G6PD depletes the NADPH available to the CYP4 group of enzymes thereby decreasing synthesis of 20-HETE. Inhibition of G6PD also activates PKG without increasing intracellular cGMP, which then inhibits 20-HETE mediated increase in mitochondrial ROS. Decrease in mitochondrial ROS leads to an increased expression of miR-143, which then inhibits ELK-1 expression. Activation of PKG by G6PD inhibition also leads to decreased ELK-1 expression, which in turn leads to a decreased expression of TNF-α. Solid lines indicate known and broken lines indicate unknown pathways.](http://ajpheart.physiology.org/10.1152/ajpheart.00961.2015)
regulated miR-143, and MitoTempol blocked 20-HETE-induced Elk-1 expression; all of this suggested that H2O2 derived from mitochondrial superoxide mediated the action of 20-HETE and this was antagonized by G6PD inhibition. Therefore, our current findings suggested that 20-HETE-induced mitochondrial superoxide production and synthetic (secretory/proinflammatory) phenotype in vascular smooth muscle cells are decreased by G6PD inhibition via activation of PKG-dependent signaling and a concurrent decrease in Erk2/MAPK1 activity.

In vascular smooth muscle cells, 20-HETE has been shown to stimulate P38K-MAPK (55) and RAS-MAPK (41) pathways that are activated by reactive oxygen species and that contribute to development of hypertension (67). 20-HETE-dependent hypertension is associated with microvascular remodeling (16, 14), a contributor to the development and maintenance of hypertension. Microvascular remodeling is promoted by a variety of stimuli resulting in structural changes including collagen synthesis and deposition, reorganization of the extracellular matrix, increased proinflammatory signaling, and altered matrix metalloproteinase activity rendering vessels stiffer and thicker and noncompliant, thus further exacerbating hypertension (29). Therefore, it is reasonable to suggest that increased 20-HETE-mediated mitochondrial superoxide generation potentially contributes to the pathogenesis of pulmonary hypertension.

In summary, the salient findings of this study are summarized in Fig. 7 and they are as follows: 1) CYP4-o-omega-hydroxylases (or 20-HETE biosynthesis is) are redox sensitive and are regulated by G6PD-derived NADPH; 2) inhibition of G6PD decreased endogenous levels of 20-HETE levels; 3) 20-HETE increased mitochondrial superoxide, which was blocked when G6PD was inhibited; 4) G6PD inhibition blocked 20-HETE-induced expression of Elk-1, which promotes the secretory phenotype of vascular smooth muscle cells; and 5) 20-HETE application to rat aorta decreased miR-143, which suppressed Elk-1 expression, and potentially promoted TNF-α secretion from vascular smooth muscle cells.

ACKNOWLEDGMENTS

A part of these results were presented at the Federation of American Societies for Experimental Biology (FASEB) meeting (Boston, MA, 2012).

GRANTS

The study was supported by intramural funds (to S. A. Gupte) and National Heart, Lung, and Blood Institute Grants R01-HL-115124 (to M. S. Wolin) P01-HL-34300 (to M. L. Schwartzman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


