20-HETE increases survival and decreases apoptosis in pulmonary arteries and pulmonary artery endothelial cells

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Dhanasekaran A, Bodiga S, Gruenloh S, Gao Y, Dunn L, Falck JR, Buonaccorsi JN, Medhora M, Jacobs ER. 20-HETE increases survival and decreases apoptosis in pulmonary arteries and pulmonary artery endothelial cells. Am J Physiol Heart Circ Physiol 296: H777–H786, 2009. First published January 9, 2009; doi:10.1152/ajpheart.01087.2008.—20-Hydroxyeicosatetraenoic acid (20-HETE) is an endogenous cytochrome P-450 product present in vascular smooth muscle and uniquely located in the vascular endothelium of pulmonary arteries (PAs). 20-HETE enhances reactive oxygen species (ROS) production of bovine PA endothelial cells (BPAECs) in an NADPH oxidase-dependent manner and is postulated to promote angiogenesis via activation of this pathway in systemic vascular beds. We tested the capacity of 20-HETE or a stable analog of this compound, 20-hydroxy-eicosa-5(2),14(2)-dieneoic acid, to enhance survival and protect against apoptosis in BPAECs stressed with serum starvation. 20-HETE produced a concentration-dependent increase in numbers of starved BPAECs and increased 5-bromo-2-deoxyuridine incorporation. Caspase-3 activity, nuclear fragmentation studies, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays supported protection from apoptosis and enhanced survival of starved BPAECs treated with a single application of 20-HETE. Protection from apoptosis depended on intact NADPH oxidase, phosphatidylinositol 3 (PI3)-kinase, and ROS production. 20-HETE-stimulated ROS generation by BPAECs was blocked by inhibition of PI3-kinase or Akt activity. These data suggest 20-HETE-associated protection from apoptosis in BPAECs required activation of PI3-kinase and Akt and generation of ROS. 20-HETE also protected against apoptosis in BPAECs stressed by lipopolysaccharide, and in mouse PAs exposed to hypoxia reoxygenation ex vivo. In summary, 20-HETE may afford a survival advantage to BPAECs through activation of prosurvival PI3-kinase and Akt pathways, NADPH oxidase activation, and NADPH oxidase-derived superoxide.

reduced nicotinamide adenine dinucleotide phosphate oxidase; reactive oxygen species; phosphatidylinositol 3-kinase; Akt; hypoxia

CYTOCHROME P-450 (CYP) ENZYMES can metabolize arachidonic acid into numerous eicosanoids, with the relative abundance dependent on the tissue and species (21). The major products in most tissues are the ω-hydroxylated metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) and regio- and stereo-specific epoxyeicosatrienoic acids. 20-HETE, a ω-hydroxylation product of arachidonic acid catalyzed by CYP4A, is a paracrine and autocrine mediator of numerous cellular processes (20, 29, 35). It is produced in vascular smooth muscle, renal, cerebral, pulmonary, mesenteric, and skeletal muscle beds and acts on the microvasculature and kidney tubules (11, 18, 22, 33, 38).

Our laboratory has recently reported that 20-HETE/CYP4 enhances reactive oxygen species (ROS) production in bovine pulmonary artery (PA) endothelial cells (BPAECs) in a manner that is associated with activation of NADPH oxidase (24). 20-HETE has been linked to ROS production in other vascular beds (19), with 20-HETE-stimulated production of ROS being reported to exert either positive or negative effects in a tissue- and concentration-specific manner (e.g., Refs. 9, 17). In addition, NADPH oxidase is proposed to have a key role in growth and migration of human coronary endothelial cells, human umbilical vein endothelial cells, and dermal microvascular endothelial cells based on blunted proliferation in cells treated with inhibitors of NADPH oxidase or transgenic mice deficient in NADPH oxidase (1, 31).

Accordingly, we tested the capacity of 20-HETE and a stable analog of this lipid to increase cell survival and decrease apoptosis in BPAECs and ex vivo PAs, and the role of NADPH oxidase-dependent ROS in this protection. In these studies, we show for the first time that 20-HETE enhances survival of PAs and BPAECs through protection from apoptosis. 20-HETE-induced prosurvival effects depend on intact phosphatidylinositol 3 (PI3)-kinase (PI3K), Akt, and NADPH oxidase pathways. Inhibition of these pathways blocks 20-HETE-induced increases in ROS production, as well as 20-HETE-induced protection from apoptosis. Similarly, inhibition of ROS production blocks 20-HETE-induced protection against apoptosis. These data provide exciting new links between ROS production, NADPH oxidase activity, and activation of PI3K prosurvival pathways. They also raise the possibility that 20-HETE may play an important role in maintaining the integrity of the pulmonary vascular bed through ROS-mediated protection against apoptosis.

MATERIALS AND METHODS

Materials. Vybrant 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) Cell Proliferation Assay Kit was obtained from Invitrogen (Carlsbad, CA, cat. no. v13154), caspase-3 Colorimetric Assay Kit from R&D Systems (Minneapolis, MN, cat. no. BF3100), LPS from E. coli 0111:B4 from Sigma-Aldrich (St. Louis, MO, cat. no. L2630), Hoechst 33342 from Invitrogen (cat. no. v13244), wortmannin from EMD Chemicals (Gibbstown, NJ, cat. no. 681675), Akt inhibitor from EMD Chemicals (cat. no. 124017), apocynin from Calbiochem (Gibbstown, NJ, no. 178385), polyethylene glycolated superoxide dismutase (PEG SOD) from Sigma-Aldrich (cat. S-9549, 685 U/mg solid, one unit inhibited rate of reduction of eNOS by 50% in a coupled system with xanthine and xanthine oxidase). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), and dihydroethidium (DHE) from Invitrogen (no. D11347). A protease inhibitor cocktail was obtained from Roche (Mannheim, Germany, cat. no. 1836 170). Protein determination kit was obtained from BioRad (Hercules, CA, cat. no. 500-0006). DNA synthesis was determined according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN, cat. no. 1647229) based on 5-bromo-2’-deoxyuridine (BrDU) incorporation into newly synthesized DNA. 20-HETE and 20-hydroxy-eicosa-5(Z),14(Z)-dienoic acid, termed 20,5,14-HEDE in this work, were synthesized in the laboratory of Dr. J. R. Falck. The structures of these compounds appear as an inset in Fig. 3A. Since both 20-HETE and analog 20,5,14-HEDE yielded similar effects on the endpoint of MTT and caspase-3 activity in pilot experiments, one or the other, but not consistently both lipids, was used for investigations in this work.

A chimeric peptide, which inhibits association of p47phox with gp91 in NADPH oxidase, was synthesized by our protein core, according to the sequence defined by Rey et al. (28) to test the contribution of NADPH oxidase to ROS production. The sequence of this peptide is [H]-R-K-R-Q-R-Q-R-C-T-R-I-R-Q-L-NH2. The sequence of the scrambled (control) peptide is R-Q-R-Q-R-C-L-R-I-T-R-Q-S-R-NH2.

Growth and culture of BPAEC. BPAEC derived from tissue obtained at a local abattoir were isolated (38) and cultured in RPMI media (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Hyclone, Logan, UT) in 100-mm2 dishes. All cells were used between passages 2 and 5 for experiments in this study.

Serum starvation experiments. BPAECs that were 60–80% confluent in 24-well plates were starved with serum-free media for 24 h. Cells were then treated with 20-HETE in concentrations ranging from 0.1 nm to 50 nm to determine cell numbers for some experiments. At the end of this time, wells were washed and cells incubated in serum-free media for 24–48 h until they were 70% confluent. They were next starved for 24–48 h. Cells were detached with trypsin and manually counted with a hemocytometer to determine cell numbers for some experiments.

Cell count method. At the end of this time, wells were washed and cells incubated in serum-free media for 24–48 h, then treated with 20-HETE in varying concentrations, vehicle, or LPS along with 20-5,14-HEDE vehicle or 20-5,14-HEDE (also 1 μM) were added for another 16 h. At the end of the experiments, cells were stained with 1 μl of Hoechst 33342 (5 mg/ml, Molecular Probes, Carlsbad, CA, V-13244) in 1 ml basal medium and incubated for 30 min. Stained cells were then washed twice with PBS (Sigma) and imaged under a fluorescent microscope using a 460-nm filter after excitation at 530 nm for assessment of nuclear fragmentation.

Hoechst stain. Cells were cultured in six-well plates to ~70% confluence. Wells were washed, and cells incubated in serum-free media containing vehicle or 20-5,14-HEDE for 8 h. After that time, vehicle or LPS along with 20-5,14-HEDE vehicle or 20-5,14-HEDE were added for another 16 h. For serum starvation experiments, cells were serum starved for 24 h, with or without application of 20-5,14-HEDE at the beginning of serum starvation and once after 16 h. At the end of the experiments, cells were stained with 1 μl of Hoechst 33342 (5 mg/ml, Molecular Probes, Carlsbad, CA, V-13244) in 1 ml basal medium and incubated for 30 min. Stained cells were then washed twice with PBS (Sigma) and imaged under a fluorescent microscope using a 460-nm filter after excitation at 530 nm for assessment of nuclear fragmentation.

MTT assays. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Sigma (St. Louis, MO, cat. no. D1385). BPAECs were seeded in 96-well plates at an initial density of 4,000/well in DMEM with 20% FBS for 48 h. After 48 h, cells were treated with 20-HETE or vehicle for 8 h, followed by reoxygenation for 16 h. The oxygen content of the culture medium was increased from 21% O2 to 95% N2 and 5% CO2 (hypoxia) or 95% air and 5% CO2 (normoxia) for 8 h, followed by reoxygenation for 16 h. The oxygen content during hypoxia (inspired O2 fraction) was continuously monitored (Pro-Ox 110, Biospherix, Redfield, NY) and did not measure above 2% at any time.

Detection of ROS by fluorescence microscopy. Primarily isolated BPAECs from passages 2–5 were used for these assays. Cells were cultured in 35-mm dishes to ~70% confluence. Inhibitors of ROS were added 30 min before loading with the fluorescent dye DHE (final concentration 10 μM). Three to four images were acquired for each dish with a Nikon Eclipse TE200 microscope equipped with fluorescence attachment (Lambda DG-4 from Sutter Instrument) and captured using a Hamamatsu digital camera C4742–95. Approximately 20 cells were randomly selected in each field, and average fluorescent intensity within operator-defined cell borders was recorded using Metamorph version 6.2 software, as previously described (24). Background fluorescence was estimated by capturing an image in an area free of cells and subtracted from the fluorescence intensity of cells on the same slide.

Statistics. Pooled data from each experiment were used to calculate the means ± SEs for control (vehicle treated) or experimental (treated with 20-HETE or 20,5,14-HEDE) samples. The data were tested for significance by one-way ANOVA followed by Holm-Sidak’s, Tukey’s, or Fisher’s post hoc tests for more than two study groups, or a Student’s t-test (for unpaired samples) using the Jandel, SigmaStat Software. Experiments with P < 0.05 were considered significant.

RESULTS

Serum-starved BPAECs exhibit enhanced survival with 20-HETE. A single application of 20-HETE to serum-starved BPAECs resulted in a concentration-dependent increase in cell numbers 18–24 h after treatments (Fig. 1). The peak response was observed in cells treated with 1 μM 20-HETE, although a significant increase in cell number was observed at 10 and 100 nM concentrations as well. Fifty micromoles of 20-HETE decreased survival of cells over that of cells treated with vehicle alone.
To examine the potential contribution of proliferation to the increase in number of cells, incorporation of BrDU into newly synthesized DNA was examined (Fig. 2). VEGF (20 ng/ml) and complete media were used as positive controls. 20-HETE increased BrDU incorporation over that of vehicle alone at concentrations of 100 nM and 1 μM. These data support the potential of 20-HETE to promote proliferation in BPAECs.

Caspase-3 activity of serum-starved cells is decreased by 20-HDE in a concentration-dependent manner. Since cell numbers can be increased both by proliferation and enhanced survival, we next tested the effect of an analog of 20-HETE, to protect against apoptosis, as detected by caspase-3 activity. The chemical structures of 20-HETE and 20-5,14-HEDE, to protect against apoptosis, as detected by caspase-3 activity. The chemical structures of 20-HETE and 20-5,14-HEDE are different. From this point forward, our studies focused on the anti-apoptotic effects of these lipids. The concentration of 20-HETE or 20-5,14-HEDE, resulting in the maximum effect on survival and protection from apoptosis (1 μM), was used to examine mechanisms of protection from apoptosis.

**Mechanism of protection against caspase-3 activation: role of NADPH oxidase.** Our data have demonstrated 20-HETE-associated activation of NADPH oxidase in BPAECs (24), as well as NADPH-dependent activation of ROS by 20-HETE in these cells. We next asked the question if protection by 20-HETE from starvation was dependent on NADPH-oxidase activity. 20-HETE-associated protection from starvation was effectively blocked by 1 μM apocynin, suggesting a requirement for intact NADPH oxidase activity for salutary effects by this lipid (Fig. 4A).

To exclude potential nonspecific effects of apocynin, we tested the ability of a peptide-based inhibitor to eliminate 20-HETE-induced protection of BPAECs from starvation (Fig. 4B). These data showed loss of the protective effect of 20-5,14-HEDE from starvation-induced increases in caspase-3 in cells pretreated with a peptide-based inhibitor of NADPH oxidase (28), although not a scrambled peptide, which served as a negative control.

**Role of PI3K in protection against starvation-associated apoptosis.** PI3K is a strong survival pathway associated with activation of NADPH oxidase in various cell systems, including normoxic lung ischemia (37). Our laboratory has previously reported 20-HETE-induced activation of PI3K, Akt, and Rac (5, 24). Accordingly, we tested the role of PI3K in 20-HETE-associated protection against caspase activity in serum-starved BPAECs (Fig. 5A). Treatment with wortmannin (100 nM) offered no protection over vehicle alone in starved cells. Furthermore, treatment with wortmannin blocked 20-HETE (1 μM) associated protection of starved BPAECs,
evoking participation of this novel signaling pathway in this injury model. To further examine the contribution of PI3K to 20-HETE-evoked protection from apoptosis, a second inhibitor of PI3K, PI-103 (100 nM), was employed in similar experiments (Fig. 5; Ref. 3). Like wortmannin, treatment with PI-103 blocked 20-5,14-HEDE-associated protection from starvation-induced increases in caspase-3.

Role of PI3K, Akt pathways, and NADPH oxidase in 20-HETE-enhanced survival, as evidenced by MTT assays. Inhibitors of NADPH oxidase, Akt, and PI3K were tested for their effects on cell survival in serum-starved BPAECs (Fig. 6). In these assays, 20-5,14-HEDE afforded protection against death, as quantitated by MTT assays. Wortmannin (100 nM), Akt inhibitor (10 μM), or apocynin (1 μM) alone had no significant effect on MTT reduction in starved cells. However, protection against starvation by 20-5,14-HEDE (1 μM) was lost in all cell cohorts treated with any of these three agents, suggesting a role for PI3K and NADPH in 20-HETE-induced cell survival, as well as caspase-3 activation. Together with Figs. 4 and 5, these data provide evidence from two different types of assays and two mechanistically distinct inhibitors to support a role for PI3K, Akt, and NADPH oxidase in protection of BPAECs from apoptosis induced by starvation.

Role of ROS in 20-HETE-induced protection against apoptosis. Our laboratory’s previous investigations have shown that 20-HETE increases superoxide and hydrogen peroxide production in BPAECs (24), an effect attributable significantly to NADPH oxidase. To determine the role of superoxide in 20-HETE-induced protection against apoptosis, caspase-3 activity was measured in BPAECs treated with 20-5,14-HEDE and simultaneously with PEG SOD (100 units) or vehicle (Fig. 7). Treatment with PEG SOD blocked 20-HETE-afforded protection against starvation-induced increase in caspase-3 activity. These data suggest a role for superoxide in protection by 20-HETE against starvation-induced apoptosis.

Wortmannin and Akt inhibition block 20-HETE-evoked superoxide production. If PI3K and Akt mediate 20-HETE-afforded protection against apoptosis via generation of superoxide, inhibition of these pathways should block 20-HETE-evoked increases in ROS. Our laboratory has previously reported that apocynin and a peptide-based inhibitor of NADPH oxidase block DHE-detected, 20-HETE-induced increases in superoxide (24). Serum starvation alone increased DHE fluorescence by 26%, consistent with activation of a prosurvival pathway (n = 120 cells per group; data not shown as a figure). All data testing the effects of inhibitors on DHE fluorescent intensity were obtained in serum-starved cells. As observed in Fig. 8, 20-HETE increases superoxide production in BPAECs, and treatment with either wortmannin or Akt inhibitor (Fig. 8) block this release. Together, these data support a role for 20-HETE-evoked, PI3K and Akt-dependent...
increases in ROS, which contribute to prosurvival effects of this agent.

LPS-induced stress: a second model of injury. To test the capacity of 20-HETE to enhance cell survival or protect against apoptosis in a second model of injury, BPAECs were pretreated with vehicle or 20-5,14-HEDE for 8 h. Then vehicle, LPS (0.5 μg/ml), or LPS + 20-5,14-HEDE were added for an additional 16 h, after which time the MTT assays were completed. Pretreatment with 20-5,14-HEDE increased survival of cells over vehicle alone in this model, while LPS decreased the same (Fig. 9). 20-5,14-HEDE offered protection of BPAECs exposed to LPS over vehicle alone.

To further study the effect of 20-5,14-HEDE in BPAECs treated with LPS, Hoechst assays were performed on the same
groups of cells as detailed above for caspase-3 experiments. Fragmentation of nuclei was absent in cells treated with vehicle or 20-5,14-HEDE alone and noted in 16% of cells treated with LPS and 2% of cells pretreated with 20-5,14-HEDE and then LPS (Fig. 10). Thus two independent assays support a survival advantage of BPAECs stressed with LPS and treated with 20-5,14-HEDE.

To examine the contribution of NADPH oxidase and PI3K to 20-HETE protection from apoptosis evoked by LPS, BPAECs stressed with LPS were treated with the gp91 peptide-based inhibitor (Fig. 11A) or PI-103 (Fig. 11B). Both of these agents blocked the protection afforded by 20-5,14-HEDE against LPS-associated increases in caspase-3 activity.

Caspase-3 activity in hypoxic ex vivo PAs: a third model of injury. We investigated the capacity of 20-HETE to protect against hypoxia-reperfusion (HR)-induced injury of ex vivo PAs. Caspase-3 activity was examined in ex vivo PAs stressed with hypoxia for 8 h followed by 16-h reoxygenation, treated with 1 μM 20-HETE, 20-5,14-HEDE, or vehicle added immediately before hypoxia exposure. HR increased caspase-3 activity over that of PAs maintained in normoxic environment, and both 20-HETE and 20-5,14-HEDE decreased caspase-3 measurement relative to those of HR-exposed vessels (Fig. 12). These data demonstrate 20-HETE- and 20-5,14-HEDE-induced protection from apoptosis in ex vivo PAs subjected to HR.

Role of PI3K in 20-HETE enhanced survival of hypoxic ex vivo PAs. To examine mechanisms through which 20-HETE afforded a prosurvival advantage in ex vivo PAs exposed to HR, we first evaluated the contribution of PI3K to salutary effects of 20-HETE in this model. Treatment with the inhibitor wortmannin (200 nM) eliminated the protection against HR afforded by 20-HETE (Fig. 13). These observations support the contribution of PI3K activation in 20-HETE-evoked protection in ex vivo PAs.

DISCUSSION

We report here for the first time that 20-HETE exerts prosurvival effects, as indicated by three separate assays, MTT uptake (enhanced cell survival), Hoechst stains (estimation of nuclear fragmentation), and caspase-3 activity (common pathway for apoptosis), in BPAECs stressed by either serum starvation or LPS. In addition, the protective effect of 20-HETE extends to ex vivo PAs exposed to hypoxia reoxygenation (HR). However, high concentrations of 20-HETE (above 1 μM) do not support cell survival and, in fact, decrease numbers of cultured BPAECs. Guo et al. (14) described proliferative, but not antiapoptotic effects of 20-HETE mediated by VEGF in human dermal microvascular cells. In a separate study, however, this group reported antiproliferative effects of HET0016 (an inhibitor of the formation of 20-HETE) in gliosarcoma cells in vivo and in vitro (15). Our studies support a pro-proliferative effect of 20-HETE in BPAECs, but we focused on the mechanisms through which this lipid promotes survival in these cells. Wang et al. (34) recently reported antiapoptotic effects of 20-HETE in PA vascular smooth muscle cells, but no mechanisms of protection other than activation of the intrinsic pathway were reported. Nilakantan et al. (26) observed enhanced ischemia-reperfusion injury in renal epithelial cells overexpressing CYP4A, an isoform that catalyzes the conversion of arachidonic acid into 20-HETE. These results may be similar to our observations of serum-starved BPAECs exposed to 50 μM 20-HETE, which decreased cell numbers.
cells treated with 20-HETE vehicle alone (ethanol). All BPAECs loaded superoxide generation. Fluorescence intensity was normalized to that of and Akt. BPAECs were loaded with dihydroethidium (DHE) to track process (31). Thus, despite well-recognized tissue injury asso-
mice, implicating both NADPH oxidase and ROS in this gration (1). Angiogenesis induced by VEGF suffused subcu-
microvascular endothelial cells require ROS derived from NADPH oxidase for proliferation and mi-
ros as well as the first antiapoptotic effects of 20-HETE in PA endothelial cells. Moreover, it is the first report of 20-HETE-associated activation of PI3K and prosurvival benefits depend-
ing on this pathway for production of ROS.

Our laboratory previously identified 20-HETE-induced stimulation of superoxide and hydrogen peroxide in BPAECs and showed that PEG SOD or PEG catalase blocks superoxide production in these cells (24). Thus we were interested to test whether ROS were required for 20-HETE-associated protection against apoptosis. This work now demonstrates that treatment of cells with PEG SOD to rapidly dismutate superoxide production stimulated by 20-HETE effectively eliminated the protective effect of this lipid on starvation-evoked apoptosis in BPAECs, providing strong evidence for the role of ROS in this process.

There is precedent for ROS either stimulating growth or promoting cell survival. ROS, including superoxide and hydrogen peroxide, signal growth and migration, activation of transcription factors, and activation of protein kinases, including ERK, p38 MAPK, and Akt (17). Nanomolar to micromolar concentrations of hydrogen peroxide stimulate proliferation and migration of bovine aortic endothelial cells (36). Human coronary and dermal microvascular endothelial cells require ROS derived from NADPH oxidase for proliferation and migration (1). Angiogenesis induced by VEGF suffused subcu-
taneously implanted sponges is deficient in Nox2 knockout mice, implicating both NADPH oxidase and ROS in this process (31). Thus, despite well-recognized tissue injury asso-
ciated with unchecked generation of ROS (10, 16), it is clear that modest levels of ROS are required for growth or protection against apoptosis.

ROS are produced from several cellular sources, including mitochondrial electron transport chain, xanthine oxidase, CYP, uncoupled nitric oxide synthase, and NADPH oxidases (27). Hyperoxia induces ROS from mitochondrial sources in pulmonary capillary endothelial cells, but subsequentlly activates NADPH oxidase through calcium-dependent and Rac-1-dependent mechanisms (4), supporting key roles for both mitochondrial and NADPH oxidase pathways in these cells. CYP4 is postulated to promote angiogenesis via NADPH oxidase-dependent mechanisms in systemic vascular beds (2, 23). NADPH oxidase catalyzes the NADPH-dependent, one-electron reduction of molecular oxygen to superoxide. In its activated form, NAPDH oxidase is a multimeric protein consisting of at least three cytosolic subunits, including p47^phox, p67^phox, p40^phox, either Rac1 or Rac2, and a membrane-associated cytochrome reductase complex consisting of gp91^phox and p22^phox (6, 13). Our studies have demonstrated that 20-HETE enhances expression and membrane association of p47^phox and gp91^phox in BPAECs (24). Moreover, 20-HETE-associated increase in ROS production is blocked by apocynin, supporting a key role for NADPH oxidase in this process (24). In the present investigations, we now provide the first evidence that 20-HETE-enhanced survival and decreased apoptosis of BPAECs stressed by starva-
tion depend on intact NADPH oxidase, since treatment of cells with apocynin or a gp91 peptide-based inhibitor largely elim-
ates protection afforded by this lipid.

The PI3K/Akt pathway provides a link between extracellu-
lar survival signals and apoptosis pathways within the cells. Activation of receptor tyrosine kinases leads to phos-

To our knowledge, the data in the present work represent the first report of proliferative actions of 20-HETE in BPAECs, as well as the first antiapoptotic effects of 20-HETE in PA endothelial cells. Moreover, it is the first report of 20-HETE-associate
phosphorylation and activation of PI3K and formation of phosphatidylinositol 3,4,5-triphosphate. Phosphatidylinositol 3,4,5-triphosphate then recruits the protein kinase Akt to the plasma membrane, where it is activated as a result of phosphorylation by phosphoinositide-dependent kinase (PDK). Akt then appears to phosphorylate a number of proteins that contribute to cell survival. Thus PI3K/Akt is a well-recognized prosurvival pathway (7).

Our published data demonstrate VEGF and 20-HETE-mediated phosphorylation of Akt in BPAECs (5). We, therefore, postulated that 20-HETE activates PI3K/Akt, which, in turn, stimulates NADPH oxidase in BPAECs. In the present study, we show that prosurvival or antiapoptotic effects of 20-HETE are blocked by wortmannin, PI-103, or an Akt inhibitor, supporting a direct role for this signaling pathway in 20-HETE protection from cell death. These data support protection from apoptosis induced by LPS by 20-5,14-HEDE. *P < 0.05 compared with 20-5,14-HEDE; **P < 0.05 relative to LPS.

Expression of 20-HETE-forming isoforms is tissue specific, and biological functions of 20-HETE outside the lung are not all salutary. For example, 20-HETE formation in cerebral vascular smooth muscle cells of spontaneously hypertensive rats is reported to contribute to the severity of oxidative stress and strokes (9). In a similar model, hypoxia leads to increased ROS formation in cerebral vascular smooth muscle, which, in turn, decreases 20-HETE formation and promotes dilation of cerebral arterioles (12). Although our data suggest a prosurvival effect of 20-HETE on BPAECs stressed by starvation, LPS, and HR, these injuries

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<tr>
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Fig. 10. Hoechst assays demonstrate protection of BPAECs injured by LPS when treated with 20-5,14-HEDE. Hoechst assays were performed on BPAECs treated with vehicle alone (control), 20-5,14-HEDE (1 μM), LPS (0.5 μg/ml) for 8 h, or LPS + 20-5,14-HEDE added simultaneously. Cells in 4 randomly selected fields of each group were assessed for fragmentation of nuclei. Top: representative images of normal and fragmented nuclei. Bottom: counts of total numbers of cells and numbers of fragmented nuclei. No fragmentation of nuclei was observed in cells treated with vehicle or 20-5,14-HEDE. LPS was associated with an increase in nuclear fragmentation. Addition of 20-5,14-HEDE to LPS reduced the percentage of fragmented nuclei to 2%. Values are means ± SE; n = 3 experiments for each group. These data support protection from apoptosis induced by LPS by 20-5,14-HEDE. *P < 0.05 compared with 20-5,14-HEDE; **P < 0.05 relative to LPS.

![Fig. 10](image_url)

Fig. 11. LPS-induced increases in caspase-3 activity depend on NADPH oxidase and PI3-kinase. A: caspase-3 activity was measured in BPAECs treated with vehicle, LPS (1 μg/ml), LPS + 20-5,14-HEDE, or LPS + 20-5,14-HEDE + gp91 peptide inhibitor (n = 5 each group). LPS resulted in an increase in caspase-3 activity, and 20-5,14-HEDE protected from LPS-induced apoptosis. Treatment with the gp91 peptide inhibitor to block NADPH oxidase eliminated 20-5,14-HEDE-evoked protection, while the scrambled peptide had no effect. B: caspase-3 measurements were obtained in BPAECs treated with vehicle, LPS (1 μg/ml), LPS + 20-5,14-HEDE, or LPS + 20-5,14-HEDE + PI-103 (100 nM final concentration; n = 6 each group). Treatment with PI-103 blocked 20-5,14-HEDE-associated protection from LPS-induced rise in caspase-3. Together, these data support a role for both NADPH oxidase and PI3-kinase in 20-5,14-HEDE protection from LPS-induced apoptosis. Values are means ± SE. *P < 0.05 relative to vehicle control in both A and B.
are believed to result in cell death by distinct mechanisms. While LPS is believed to initiate apoptosis via activation of Fas ligands, HR causes cell loss through not only apoptosis, but also oncosis and necrosis (30). Our data support a role for PI3K and NADPH oxidase in 20-HETE protection from LPS. The role of other signaling pathways (e.g., oncosis) in cells stressed with LPS or hypoxia and treated with 20-HETE has not been examined.

In conclusion, these studies report, for the first time, antiapoptotic effects of 20-HETE against three forms of injury, which are serum deprivation, LPS, and HR in BPAECs and ex vivo PAs. Survival of endothelial cells depends on ROS, NADPH oxidase, and PI3K and Akt. We hypothesize (Fig. 14) that 20-HETE activates PI3K and Akt first, which, in turn, activate NADPH oxidase to produce ROS. Based on our data, it is unclear which ROS product is critical to the prosurvival properties of 20-HETE, but it may be hydrogen peroxide, which has strong pro-proliferative properties (17). These functions suggest a potential role for CYP4 in regulating growth and survival of endothelial cells, which could have important physiological and pathophysiological implications in disorders such as pulmonary hypertension, recovery from a severe pneumonia, susceptibility to hypoxic lung injury, and others. Further studies to clarify the interaction of pro- and antisurvival signaling pathways triggered by 20-HETE, and the role of endogenous 20-HETE, are needed.

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