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Research Article

Tissue protection and endothelial cell signaling by 20-HETE analogs in intact *ex vivo* lung slices

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ABSTRACT

The capacity to follow cell type-specific signaling *in intact lung* remains limited. 20-hydroxyeicosatetraenoic acid (20-HETE) is an endogenous fatty acid that mediates signaling for a number of key physiologic endpoints in the pulmonary vasculature, including cell survival and altered vascular tone. We used confocal microscopy to identify enhanced reactive oxygen species (ROS) production in endothelial cell (EC)s in intact lung evoked by two stable analogs of 20-HETE, 20-5,14-HEDE (20-*h*ydroxy*e*icosa-5(Z),14(Z)-*di*enoic acid) and 20-5,14-HEDGE (N-[20-*h*ydroxy*e*icosa-5(Z),14(Z)-*di*enoyl]glycin*e*). These analogs generated increased ROS in cultured pulmonary artery endothelial cells as well. 20-HETE analog treatment decreased apoptosis of pulmonary tissue exposed to hypoxia-reoxygenation (HR) *ex vivo*. Enhanced ROS production and apoptosis were confirmed by biochemical assays. Our studies identify physiologically critical, graded ROS from ECs in live lung tissue *ex vivo* treated with 20-HETE analogs and protection from HR-induced apoptosis. These methodologies create exciting possibilities for studying signaling by stable 20-HETE analogs and other factors in pulmonary endothelial and other lung cell types in their native milieu.

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Introduction

Reactive oxygen species (ROS) have been implicated as mediators of cellular proliferation/survival as well as cell death, depending on the amount, source and trigger. In pulmonary artery endothelial cells, one trigger for increased ROS and enhanced survival is treatment with the cytochrome P450 metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) or analogs of the same. Although

Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; BPAEC, Bovine pulmonary artery endothelial cell; DCF, Dichlorofluorescein; DHE, Dihydroethidium; DMEM, Dulbecco's modified essential media; EC, Endothelial cell; FITC, Fluorescein isothiocyanate; LDL, Low density lipoprotein; PECAM, Platelet endothelial cell adhesion molecule; PEG-SOD, Polyethylene glycol coupled superoxide dismutase; PBS, Phosphate buffered saline; HR, Hypoxia-reoxygenation; ROI, Region of interest; ROS, Reactive oxygen species; TUNEL, Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling

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20-HETE has pro-apoptotic actions in some organ systems [1,2], we have extensive evidence demonstrating anti-apoptotic action in lung tissue [3–5]. In cultured pulmonary artery endothelial cells (ECs), the pro-survival effects of this lipid are attributable to modest increments in superoxide production which appears to derive in part from NADPH oxidase and ROS [4,5]. However, studies with isolated cells provide little information about the physiological interactions that occur *in situ* when endothelial and other lung cells are juxtaposed. Therefore we developed a novel model to examine the effect of 20-HETE analogs on superoxide production from pulmonary artery and capillary ECs with intact physico-chemical interactions to other cell types. We describe a method for preparation of rodent lung slices to detect endothelial-specific effects of ROS production using dihydroethidium (DHE) fluorescence and CD31 (platelet endothelial cell adhesion molecule-1, or PECAM) staining. Previous models described by others [6,7] did not permit cell-type specific quantification of ROS production. We extended our model to study protection by 20-HETE analogs from apoptosis induced by *ex vivo* hypoxia-reoxygenation in intact lung slices. This approach opens new avenues for unraveling the functional significance of 20-HETE analog-stimulated pulmonary endothelium-derived ROS in lung disease, and allows for direct visualization of local signaling events within the endothelial microenvironment.

Materials and methods

20-HETE (*h*ydroxy*e*icosa*t*etra*e*noic acid), 20-5,14-HEDE (20-*h*ydroxy*e*icosa-5(Z),14(Z)-*die*noic acid) and 20-5,14-HEDGE (N-[20-*h*ydroxy*e*icosa-5(Z),14(Z)-*d*ienoyl]glycin*e*) were synthesized in the laboratories of Dr. John Falck. Fig. 1 shows the structures of these compounds. The 20-HETE analogs have advantages over native 20-HETE in that they are not subject to autoxidation. Varying degrees of autoxidation increase variability in experimental endpoints of interest. In addition, the analogs are not substrates for cyclooxygenase or lipoxygenase. Non-enzymatic oxidation products of arachidonic acid and polyunsaturated fatty acids such as isoprostanes have important effects on lung biology [8], as do cyclooxygenase products of 20-HETE [9]. Both analogs had qualitatively similar effects on DHE fluorescence in cultured PAECs and lung slices (see Fig. 2) as well as endpoints of interest in our published studies of 20-HETE in the lung [4,5,10]. For each set of experiments, one or the other analog was used as identified in the text and graphs.

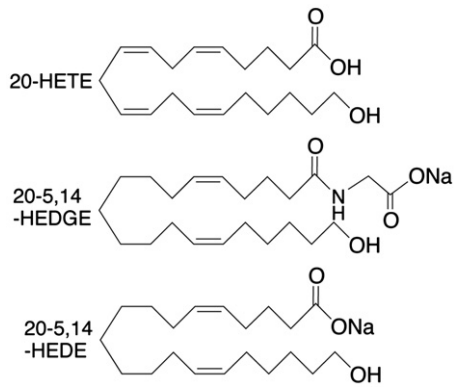

Fig. 1 – Chemical structure of 20-HETE, 20-5,14-HEDE and 20-5,14-HEDGE.

Isolation and preparation of pulmonary artery endothelial cells (PAECs)

Bovine PAECs were isolated in a manner previously described by us [3]. Cells were grown to 80% confluence and made quiescent by removal of serum for six hours. Cells were used between passages 2 and 5, and loaded with a final concentration of 10 μM DHE for 10 min before washing. They were stimulated with vehicle, 20-HETE or analogs in final concentrations of 1 μM. BSA (0.1%) was added as a lipid carrier before fluorescence imaging.

Preparation and staining of lung slices with DHE and PECAM

Animal protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Lungs were harvested from adult female rats Wistar (WAG/Rij/Cmcr) under deep anesthesia and rinsed in phosphate buffered saline (PBS). Unfrozen, intact lung tissue ($\sim 5 \times 5 \times 5$ mm) was held in place by 2% agarose blocks, then 300 μm sections were obtained on a transverse plane with a microtome (OTS-4000, Electron Microscopy Sciences, Hatfield, PA). Lung sections were collected in iced PBS, then transferred to Dulbecco's modified essential media (DMEM), where they were incubated with polyethylene glycol coupled superoxide dismutase (PEG-SOD; cat# S9549 Sigma) or vehicle, then 10 μM dihydroethidium (DHE) for 10 min at 37 °C. After washing and transferring to fresh DMEM, lung slices were treated with vehicle (ethanol) or 20-HETE analog (1 μM) for 15 min. After a final rinse in PBS, lungs were imaged straight away or fixed for 90 min with 4% paraformaldehyde and PBS. Fixed sections were treated with primary antibody for CD31 (cat# 550300, BD Pharmingen) and examined in a confocal microscope. To confirm specificity of the stain, some thick sections were frozen in OCT compound and 10 μm sections prepared for imaging as below.

Fluorescence imaging

For some experiments as indicated in the text, a Nikon Eclipse TE2000-U with a camera attachment (Qimaging QCAM FAST394) was used to acquire fluorescent images. For confocal microscopic data acquisition, an inverted microscope (Zeiss Axiovert 510, Carl Zeiss), equipped with a three-line (488, 569, and 647 nm) argon-krypton laser, was used for excitation of the DHE and fluorescein isothiocyanate (FITC) labels. For DHE, a 610–650-nm band pass filter was employed with excitation at 488 nm using an argon laser; for FITC, a 505–550 nm band pass emission filter was used, with excitation at 488 nm using an argon laser from each tissue slice. Rhodamine-tagged terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) positive cells were identified by excitation at 555 nm and detection at 580 nm. To avoid cross-talk between the fluorescence channels, probes were scanned sequentially.

Imaging experiments were performed at room temperature. Detector and laser settings were held constant across all samples within individual experiments, with control and experimental