Original Article

Optical cryoimaging of mitochondrial redox state in bronchopulmonary-dysplasia injury models in mice lungs

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Background: Bronchopulmonary dysplasia (BPD) is a major cause of morbidity and mortality in premature infants exposed to high levels of oxygen. This is mainly attributed to increased oxidative stress and angiogenesis defects impacting lung alveolarization.

Methods: Here we use optical imaging to investigate the role of Bcl-2 in modulation of oxidative stress and angiogenesis and pathogenesis of BPD. Cryoimaging of the mitochondrial redox state of mouse lungs was applied to determine the metabolic state of the lungs from Bcl-2 +/+ (control), Bcl-2-deleted in the endothelium (Bcl-2 VE-cad) and Bcl-2-deficient (Bcl-2 -/-; global null) using mitochondrial metabolic coenzymes NADH (Nicotinamide Adenine Dinucleotide), and FADH2 (Flavin Adenine Dinucleotide) as the primary electron carriers in oxidative phosphorylation.

Results: We observed a 47% and 26% decrease in the NADH redox in Bcl-2 deficient lungs, Bcl-2 -/- and Bcl-2 VE-cad, respectively.

Conclusions: Thus, Bcl-2 deficiency is associated with a significant increase in oxidative stress contributing to reduced angiogenesis and enhanced pathogenesis of BPD.

Keywords: Bcl-2; lung tissue; mitochondrial redox; optical imaging

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung condition that affects premature infants who receive supplemental oxygen (hyperoxia) or ventilator support for long periods of time. Studies have shown that arrest of alveolar development is a hallmark of BPD caused by either oxygen or mechanical ventilation. We have observed significant abnormalities in lungs prepared from Bcl-2 null mice perhaps as a result of increased oxidative stress and reduced angiogenesis (1). Our hypothesis is that oxidative stress plays a key role in the development of vascular dysfunction associated with BPD. We used optical cryoimaging to investigate the mitochondrial redox state of the tissue related to oxidative stress and pathogenesis of BPD.

Anatomical and functional information of tissue can be obtained by fluorescence imaging techniques via intrinsic fluorophores or exogenous tagged proteins (2) and are used to probe tissue redox state and energy homeostasis in various organs with a high sensitivity and specificity for discriminating between diseased and non-diseased tissue (3). These fluorescence images are able to monitor tissue metabolic state, as an indicator of cellular oxygen consumption (4,5).

NADH and FAD (oxidized form of FADH2), two

mitochondrial metabolic coenzymes and the primary electron carriers in oxidative phosphorylation, are intrinsic fluorophores and can be monitored using fluorescent imaging. It has been shown that the ratio of these fluorophores, NADH/FAD, called the mitochondrial redox ratio (RR) (6-9), is a marker of the mitochondrial redox and metabolic state of tissue and a change in RR is an index of a change in lung tissue bioenergetics. Here we tested whether deficiency in Bcl-2, an anti-apoptotic protein with important role in angiogenesis (10), results in increased oxidative stress and attenuation of lung angiogenesis contributing to PBD.

Methods

As our injury model, lungs from three groups of mice were studied: Bcl-2 +/+, Bcl-2 -/- (global Bcl-2 null) and Bcl-2 VE-cad (Bcl-2 only deleted in the endothelium). Bcl-2 +/+ lungs were used as control and Bcl-2 VE-cad and Bcl-2 -/- mice were used as potential models of BPD. The mice were sacrificed at 3 weeks of age.

Lung tissue metabolic state was preserved by rapid freezing, immediately after harvesting the tissue, in chilled isopentane (2-methyl butane, Fisher Scientific, IL) within liquid nitrogen (LN2, –196 ℃) then embedded in a customized fluorescent-free black mounting medium for cryo fluorescence imaging.

Cryoimaging or Low-temperature fluorescence imaging provides 3-D fluorescence images of cryopreserved intact organs. Imaging in lower temperatures (–40 ℃) guaranties a higher quantum yield of fluorescence of NADH and FAD as compared to room temperature (11-13). Cryoimager is an automated image acquisition instrument consisted of hardware and software designed to acquire fluorescence images of tissue sections. Image acquisition and the instrument have been previously described (14). Briefly each sample is sequentially sliced using a computer controlled microtome and NADH and FAD fluorescent images is captured for each slice using a CCD camera.

For the Image Processing step, the composite images were created using all the image slices for each lung, for both NADH and FAD signals. The ratio of NADH and FAD (NADH/FAD) was calculated voxel by voxel, using Matlab. For each lung, a histogram of RR values was created, and the mean (or first moment) of this histogram was calculated for the whole volume of the tissue according to Eq. [1].

$$Mean = \frac{1}{N_x \times N_y \times N_z} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \sum_{k=1}^{N_z} Lung_{Vol(i,j,k)} \quad [1]$$

Statistical comparisons were also carried out on a population of N=3 for control and N=3 for Bcl-2 VE-cad and N=4 for control and N=9 for Bcl-2 -/- mice lungs using a one-tailed student's *t*-test, with $P<0.05$ for each group as the criterion for statistical significance.

Results

Figure 1A shows the 3-D rendering of NADH and FAD fluorescence signals and their ratio (RR = NADH/FAD) from representative lungs of each of the three groups (Bcl-2 VE-cad *vs.* Bcl-2 +/+ on top and Bcl-2 -/- *vs.* Bcl-2 +/+ on bottom). As expected, mice with global Bcl-2 null and Bcl-2 VE-cad showed a decreased NADH signal and an increased FAD signal and as a result decreased RR in respect to the control mice (26% decrease for Bcl-2 VE-cad and 47% for Bcl-2 -/-) which implies generation of more ROS and oxidative stress. Bcl-2 -/- mice also had more ROS and oxidative stress compared with Bcl-2 VE-cad. *Figure 1B* shows histograms of RR for the lungs. The mean values of these histograms suggest a more reduced mitochondrial redox state for Bcl-2 +/+ lung, and more oxidized mitochondrial redox state for both Bcl-2 VE-cad and Bcl-2 -/-. *Figure 1C* shows the average ± SE (standard errors) of the mean values of the RR histograms for the three groups of mice, which shows a significant decrease ($P<0.021$) in the NADH redox in Bcl-2 VE-cad and Bcl-2 -/- lungs.

Discussion and conclusions

We have previously demonstrated the utility of cryoimaging for evaluating the redox status of tissue mitochondrial coenzymes NADH and FAD in intact lungs in another model of BPD (combining injuries due to ventilation with elevated oxygen concentration and bacterial infection) (15). We have shown that the RR, NADH/FAD, is an index of lung tissue mitochondrial redox state, and is an important determinant of mitochondrial bioenergetics. Here we have shown that mice lacking Bcl-2 demonstrate increased oxidative stress as seen in BPD phenotype. Bcl-2 is a key mediator of downstream events that occur in response to both pro- and anti-angiogenic factors, including VEGF and thrombospondin-1 (TSP1), respectively (16). The important role Bcl-2 plays during angiogenesis is demonstrated by the

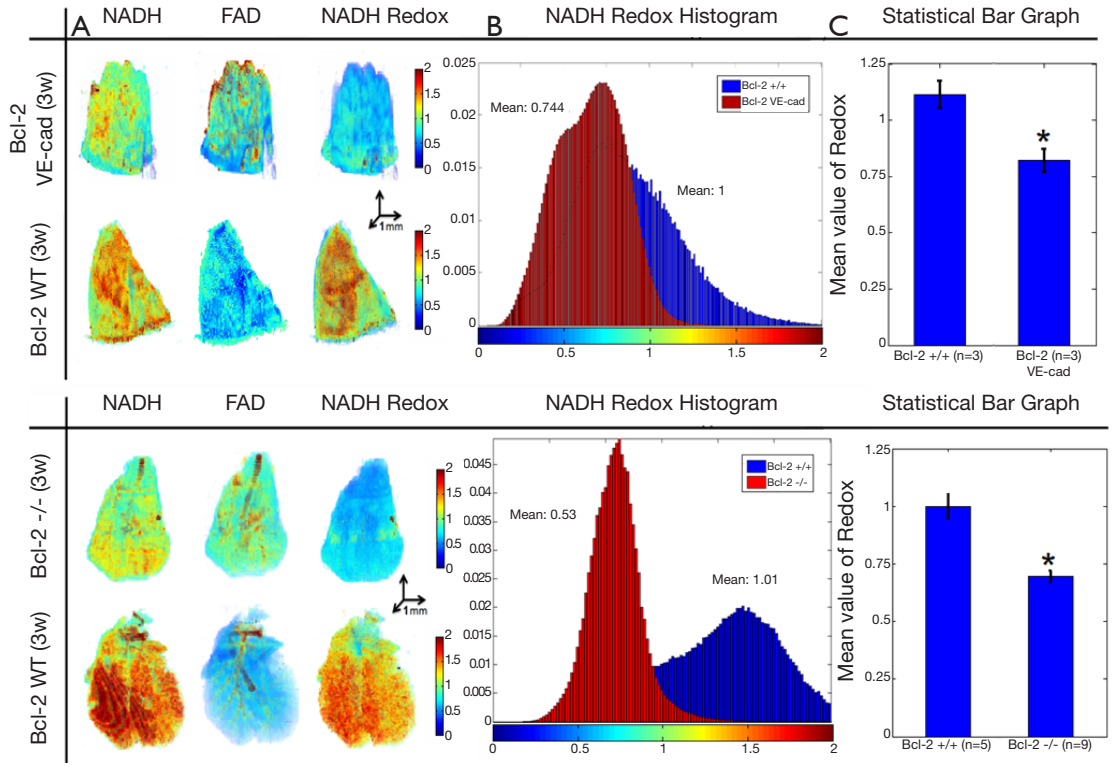

Figure 1 (A) Fluorescence images of from left to right NADH, FAD and NADH redox in a Bcl-2 VE-cad *vs*. Bcl-2 +/+ mouse lung on top and Bcl-2 -/- *vs*. Bcl-2 +/+ on the bottom; (B) histogram of control (blue) and Bcl-2 VE-cad lung (red) on top and control (blue) and Bcl-2 -/- lung (red) on bottom; (C) bar graph showing the means and standard errors of the mean value of mitochondrial redox ratio of control and Bcl-2 VE-cad on top and control and Bcl-2 -/- on bottom. *, shows statistical significant difference P<0.021.

inability of Bcl-2 -/- endothelial cells to undergo capillary morphogenesis and sprouting angiogenesis (17).

A clearer understanding of mitochondrial dysfunction and the role Bcl-2 plays in this process is critical for elucidating the role of mitochondrial bioenergetics in pulmonary developmental arrest and can be further used in prevention of BPD-like injuries. Our studies show that deficiency of Bcl-2 in the endothelium is only partially responsible for increased oxidative stress. The identity of additional cellular components to increased oxidative stress in the global null mice awaits further investigation.

Other endogenous fluorophores in the tissue including collagen and elastin would not be expected to contribute in variations of mitochondrial redox state (18,19). Contribution of cytosolic NADPH, which has the same fluorescence characteristics as NADH, to the NADH fluorescent signal is considered to be small (20) since its concentration and quantum yield is much smaller than NADH (21,22).

NADH and FAD data provide information regarding tissue redox and mitochondrial bioenergetics, a truer and more sensitive early measure of organ function. Because NADH and FAD signals can be detected through fiber optic probes placed on the surface of the lung, RR data could be obtained either intraoperatively or through tube thoracostomies (frequently placed for clinical indications in patients with severe lung injury). Our studies support the capacity of fluorescence imaging to detect pulmonary oxidative injury, and set the stage for *in vivo* studies and further translation to clinical arenas.

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