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## Natural antioxidant dihydroxybenzyl alcohol blocks ritonavir-induced endothelial dysfunction in porcine pulmonary arteries and human endothelial cells

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

Sarah M. Weakley<sup>ABCDE</sup>, Jun Jiang<sup>ABCD</sup>, Jianming Lü<sup>ABCD</sup>, Xinwen Wang<sup>ABCD</sup>, Peter H. Lin<sup>AD</sup>, Qizhi Yao<sup>AD</sup>, Changyi Chen<sup>ABCDEFG</sup>

Molecular Surgeon Research Center, Division of Vascular Surgery and Endovascular Therapy, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX, U.S.A.

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### Summary

#### Background:

Patients with HIV have an increased incidence of pulmonary artery hypertension. This study was designed to determine if the naturally occurring antioxidant dihydroxybenzyl alcohol (DHBA) could counteract the deleterious effects of ritonavir (RTV), an HIV-protease inhibitor known to impair endothelial function and increase oxidative stress.

#### Material/Methods:

Antioxidant assays were performed on DHBA in a cell free system. Glutathione (GSH) levels were measured in human pulmonary artery endothelial cells (HPAEC) to determine the effect of DHBA on the level of oxidative stress in cells treated with RTV. Myograph analysis was performed on porcine pulmonary artery (PA) rings after treatment with RTV and/or DHBA. Likewise, reactive oxygen species (ROS) production was assessed in porcine PA rings after RTV +/- DHBA using a lucigenin reaction. Immunohistochemical staining for endothelial nitric oxide synthase (eNOS) was also performed in porcine PAs treated as above.

#### Results:

DHBA demonstrated significant antioxidant activity in a cell free system that surpassed that of vitamin C. Also, treatment with DHBA reduced RTV-induced reduction in endothelium-dependent vasorelaxation and eNOS staining and increased superoxide anion levels. Meanwhile, there was a reversal in RTV-induced oxidative stress leading to reduced GSH levels in HPAECs after treatment with DHBA.

#### Conclusions:

These findings suggest that the naturally occurring antioxidant DHBA reduces the impairment of vasomotor functions caused by RTV in porcine PAs and reduces oxidative stress caused by RTV in HPAEC and porcine PA rings. This study indicates that DHBA may have clinical applications in the prevention or treatment of antiretroviral drugs-associated vascular complications in patients with HIV.

#### key words:

**dihydroxybenzyl alcohol • ritonavir • antioxidant • pulmonary artery hypertension • oxidative stress • endothelial dysfunction • endothelial nitric oxide synthase**

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#### Author's address:

Changyi (Johnny) Chen, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, One Baylor Plaza, Mail stop: BCM391, Houston, TX, U.S.A., e-mail: jchen@bcm.tmc.edu

## BACKGROUND

In today's era of highly active antiretroviral therapy (HAART), patients with human immunodeficiency virus (HIV) are living longer due to improved immune function and fewer opportunistic infections. However, these patients have an increased incidence of cardiovascular and pulmonary disease, including pulmonary artery hypertension (PAH). PAH can be idiopathic, familial, or "associated" PAH; HIV-associated PAH is a well established complication of infection [1]. In the early 1990s, the prevalence of HIV-associated PAH was 0.5%, and this has been relatively stable since the subsequent advent of HAART therapy [2]. Meanwhile, there is evidence that treatment with HAART does not prevent HIV-associated PAH in infected patients, and mortality in patients with HIV-associated PAH is most often related to the PAH rather than other complications from HIV infection [3].

It has been widely accepted that the primary pathobiologic culprit in the development of pulmonary artery hypertension is the endothelial cell [4,5]. Endothelial dysfunction in PAH involves changes in EC permeability and the bioavailability of molecules important in signaling, differentiation, transcription, and cell growth, such as nitric oxide (NO), which is an important vasodilatory mediator; reduced expression, activity and/or activation of endothelial NO synthase (eNOS) can reduce NO bioavailability [6]. While the specific etiology of HIV-associated PAH has not been definitively established, the inflammatory bloodstream environment created by infection or specific viral proteins has been implicated [1].

HIV protease inhibitors such as ritonavir (RTV) have been shown to impair endothelial functions and increase oxidative stress in porcine pulmonary arteries and human pulmonary artery endothelial cells (HPAECs) [7]. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are generated in normal human cells during inflammatory states and cellular respiration; these molecules play important roles in signaling and other biologic functions [8]. Meanwhile, ROS in excess can be very damaging to cells, and oxidative stress has been implicated in many vascular diseases. It is likely that the effects of RTV on endothelial oxidative stress contribute to vascular disease formation.

Consumption of a "Mediterranean diet" consisting of antioxidant-rich foods has been associated with a decreased risk of cardiovascular disease and stroke [9,10], which are diseases related to loss of endothelial integrity and endothelial dysfunction [11]. The Mediterranean diet involves the use of antioxidant-rich red wine and olive oil, coupled with nutrition from fruits, whole grains, vegetables, beans, and legumes rather than red meat. Olive oil is a staple of the Mediterranean diet, and polyphenolic compounds found in virgin olive oils are not just responsible for their bitterness and pungency but have important antioxidant properties and are powerful free radical scavengers [12,13].

The objective of this study was to examine the effects of dihydroxybenzyl alcohol (DHBA), a naturally occurring antioxidant found in the phenolic fraction of virgin olive oil [12], on the deleterious effects of RTV on endothelial dysfunction. We hypothesized that treatment of porcine pulmonary arteries and HPAECs with DHBA would reduce the

vasomotor dysfunction and increase in oxidative stress observed after treatment with RTV.

## MATERIAL AND METHODS

### Chemicals and reagents

RTV was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. RTV was dissolved in DMSO. DHBA was obtained from TCI America (Portland, OR) and was dissolved in water. For immunohistochemistry, the ABC kit from Vector laboratories (Burlingame, CA) was used. Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies and the enhanced chemiluminescence kit were obtained from Amersham Life Sciences (Buckinghamshire, UK). The biotinylated horse anti-mouse IgG and avidin-biotin complex kit were obtained from Vector labs (Burlingame, CA). Glibco antibiotic-antimycotic was obtained from Invitrogen. Clonetics EGM-2 SingleQuots growth factors were used for cell culture (Lonza, Walkersville, MD). GSH-Glo Glutathione Assay was obtained from Promega (Madison, WI). Thromboxane A<sub>2</sub> analogue U46619, bradykinin, sodium nitroprusside (SNP) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Mediatech Inc. (Manassas, VA). Lucigenin was obtained from Sigma-Aldrich.

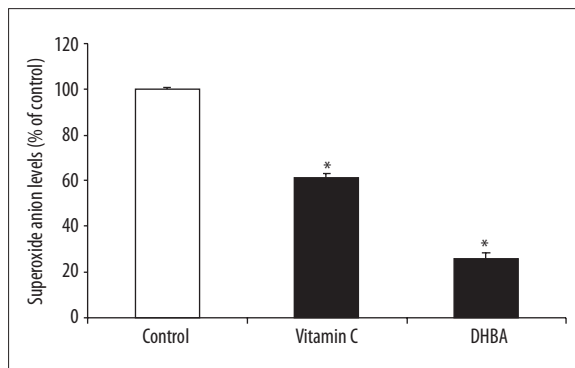
### Antioxidant activity assays

Superoxide (O<sub>2</sub><sup>-•</sup>) scavenging activity was assayed in the xanthine-xanthine oxidase system and determined by the inhibition of reduction of nitroblue tetrazolium (NBT) to form blue formazan, which has absorption at 560 nm [14]. O<sub>2</sub><sup>-•</sup> production and xanthine oxidase activity were measured as NBT reduction (at 560 nm) and uric acid production (at 295 nm), respectively. The ability of DHBA to scavenge the stable DPPH radical was measured optically by monitoring the decreases of their absorptions at 429 nm, according to the literature [15].

### Myograph analysis

The myograph system used in this experiment has been previously described [7,16]. Briefly, fresh porcine lungs were harvested from the healthy young pigs at the time of slaughter, preserved in ice-cold PBS during transport to the laboratory, and then the third division branches of the pulmonary arteries were isolated. These were cut into 3–4 mm rings. In each treatment group, 10 rings were treated as follows and incubated in DMEM with 1% antibiotic/antimycotic for 18–24 hours: no treatment (control); RTV (15 μM); DHBA (10 μM); and RTV with increasing concentrations of DHBA (1 and 10 μM). Then, the rings were suspended between the wires of the organ bath chamber (Multi Myograph system 700MO; Myo Technology, Aarhus N, Denmark) in 6 mL of Kreb's solution.

Myograph tension analysis was performed as follows. The pulmonary arteries were stimulated with thromboxane A<sub>2</sub>-analogue U46619 and the maximal contraction was recorded. Then, endothelium-dependent vasorelaxation was assessed by treatment with increasing concentrations of bradykinin. Finally, the rings were treated with sodium nitroprusside at 10<sup>-6</sup> M to obtain endothelium-independent vasorelaxation.



**Figure 1.** Superoxide scavenging ability of DHBA in the cell free system. The ability of DHBA to scavenge superoxide anion ( $O_2^{\cdot-}$ ) was assayed in the xanthine-xanthine oxidase system; superoxide anion production was measured as reduction of nitroblue tetrazolium (at 560 nm). DHBA had a significantly larger effect on lowering superoxide anion levels than that of vitamin C (74% vs. 39%).  $n=3$ .

### Cell culture

HPAEC were purchased from Lonza (Walkersville, MD). Cells used at passage 4 to 6. Once they reached 70–80% confluence, they were treated with RTV and/or DHBA. Then, glutathione assay was performed.

### Glutathione assay

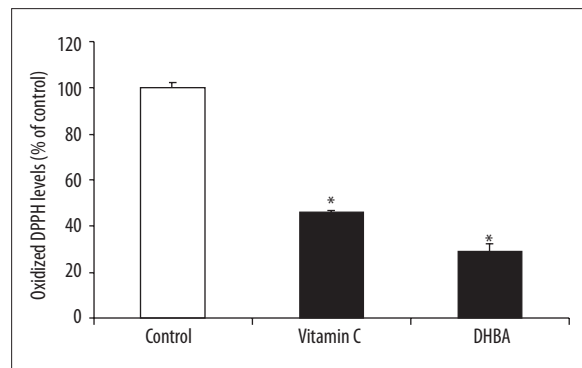
Glutathione assay measures glutathione (GSH) levels to determine oxidative stress in cells. HPAECs were treated in the following groups: no treatment (control), RTV (7.5  $\mu$ M) only, DHBA (10  $\mu$ M) only, or RTV (7.5  $\mu$ M) after pre-treatment for one hour with DHBA (10  $\mu$ M or 50  $\mu$ M). GSH levels were measured using a luciferase reaction (GSH-Glo Glutathione assay).

### Vessel ring superoxide assay

Porcine pulmonary arteries were harvested as described and kept in ice-cold PBS until arrival in the lab. There, they were cut into 5–6 mm rings and incubated in DMEM overnight with the following treatments: no treatment (control); RTV (15  $\mu$ M); DHBA (10  $\mu$ M); and RTV with DHBA (1  $\mu$ M and 10  $\mu$ M). The rings were rinsed in HEPES solution and then opened, cut into 5×5 mm pieces, measured precisely, and placed endothelium-side down in an assay tube with 25  $\mu$ L of lucigenin solution (1 mM) in 500  $\mu$ L HEPES buffer. The vessels were then subjected to luminometer analysis, and the relative light units (RLU)/s/mm<sup>2</sup> was calculated, representing ROS generation.

### Porcine eNOS immunohistochemistry

Porcine pulmonary artery rings were collected as described above. The rings were treated as above, rinsed in PBS, and then fixed in formalin. They were then embedded in paraffin, sliced into 5  $\mu$ m-thick cross sections, and mounted on slides. They were then treated with 0.3%  $H_2O_2$  in PBS, rinsed in PBS, and subjected to blocking serum using the ABC kit. Then, they were incubated in primary antibody against eNOS, rinsed in PBS, and incubated in secondary



**Figure 2.** Antioxidant index of DHBA in the cell free system in comparison with Vitamin C. The ability of DHBA to scavenge the DPPH radical was measured by the reduction in oxidized DPPH levels. DHBA had a significantly greater antioxidant activity than vitamin C in this system (71% vs. 54%).  $n=3$ .

antibody (ABC kit). Finally, they were rinsed again in PBS, incubated in ABC reagent again, then incubated in DAB, rinsed, counterstained with hematoxylin and eosin, covered, and inspected under light microscopy.

### Statistical analysis

All data are presented as the mean  $\pm$  SEM. Differences among three or more groups were analyzed using one-way analysis of variance. Student's t-test was used for comparison between two groups. A P value of  $<0.05$  was considered significant.

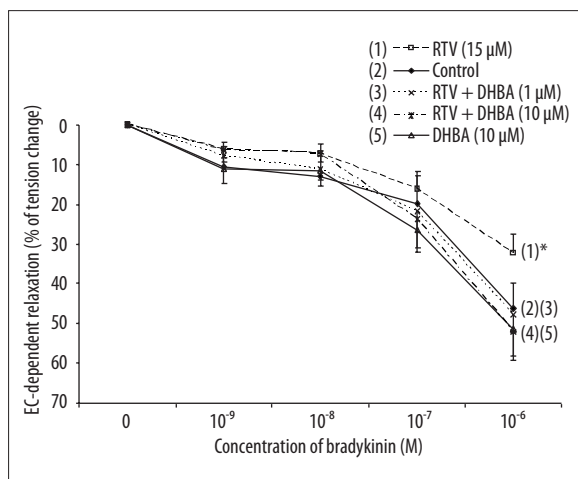
## RESULTS

### DHBA has strong antioxidant activities in the cell free system

The redox state of the vascular endothelium is a major determinant of vessel integrity, function, and health. Imbalance in the homeostasis between endogenous antioxidants and ROS can lead to endothelium injury and dysfunction. Small exogenous antioxidants may plan an important role in protecting the endothelium from oxidatives stress [17]. We tested the antioxidant activity of DHBA in a cell-free system against Vitamin C and determined that DHBA has a more potent antioxidant effect on superoxide anion levels; DHBA led to a 74% reduction, compared to 39% reduction with Vitamin C treatment. This indicates an enhanced superoxide scavenging capacity in comparison to Vitamin C (Figure 1). We also assayed DHBA in comparison to Vitamin C using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The DPPH free radical becomes reduced in the presence of antioxidants, leading to a color change that is quantifiable, being stoichiometric with respect to the number of electrons captured. The DHBA, again, had a more potent antioxidant effect than Vitamin C (71% vs. 54%) (Figure 2).

### DHBA blocks RTV-induced endothelial dysfunction in porcine pulmonary arteries

RTV is known to cause endothelial dysfunction, and it has been shown experimentally to impair endothelium-dependent relaxation in both porcine coronary and pulmonary arteries [7,18]. As these effects appear to be mediated by

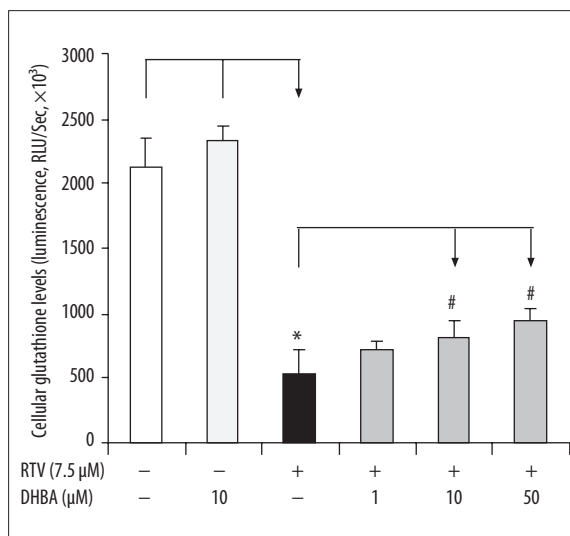


**Figure 3.** Effects of DHBA on the RTV-induced endothelial dysfunction in porcine pulmonary artery rings. Fresh porcine PA rings were harvested and incubated overnight after treatment with RTV (15  $\mu$ M), DHBA (10  $\mu$ M) or RTV and DHBA (1 or 10  $\mu$ M) and endothelium-dependent vasorelaxation was measured in comparison to a no-treatment control group. RTV treatment reduced endothelial-dependent vasorelaxation, while treatment with DHBA ameliorated this effect in a dose dependent manner.  $n=10$ ,  $P<0.05$ .

eNOS downregulation and increased markers of oxidative stress [7,18], we sought to determine whether concomitant treatment of porcine pulmonary artery rings with antioxidant DHBA could reduce this observed impairment in endothelial function. When the porcine pulmonary artery rings were treated with 15  $\mu$ M RTV, bradykinin-dependent relaxation was reduced, as expected (Figure 3). However, when rings were also treated with DHBA, there was a significant increase in vessel ring relaxation after treatment with bradykinin (which is endothelium-dependent relaxation in our model). For example, endothelium-dependent vasorelaxation was reduced by 30.5% in pulmonary artery rings treated with RTV (15  $\mu$ M) compared with controls in response to bradykinin (10<sup>-8</sup> M) (Figure 3,  $P<0.05$ ); this effect was reversed when the rings were incubated in RTV 15  $\mu$ M along with DHBA at 1  $\mu$ M and 10  $\mu$ M, with relaxation increased by 48% and 62%, respectively in response to bradykinin (10<sup>-8</sup> M,  $P<0.05$ ). There was no significant difference in endothelium-independent vasorelaxation within these groups after treatment with sodium nitroprusside.

#### DHBA blocks RTV-induced oxidative stress in HPAECs

Glutathione peroxidase is an important endogenous regulator of oxidative stress in cells; it functions as an antioxidant by reducing free hydrogen peroxide to water. In doing so, reduced glutathione (GSH) is consumed and converted to its oxidized form, glutathione disulfate (GSSG); the amount of GSH in a system, then, is inversely proportional to the level of oxidative stress in the cells. We performed a glutathione assay on HPAEC that relied on the conversion of a luciferin derivative into luciferin in the presence of GSH. Overnight treatment of HPAEC with RTV (7.5  $\mu$ M) led to a reduction in GSH levels by 75% ( $P<0.05$ ), indicating increased oxygen

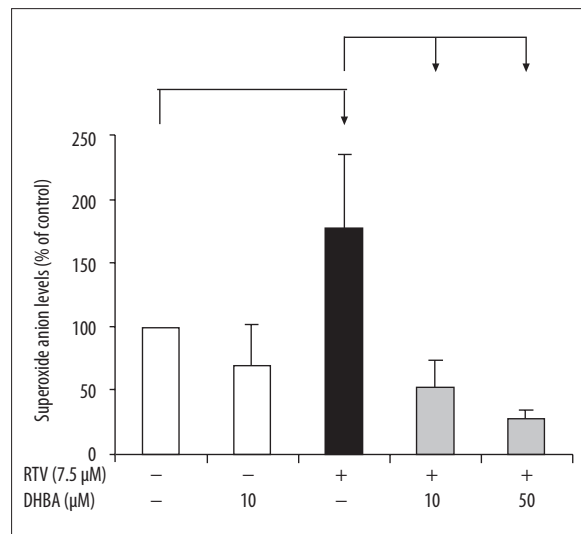


**Figure 4.** Effects of DHBA on the RTV-induced increase of cellular glutathione (GSH) levels in HPAECs. GSH levels were measured in HPAEC after treatment with RTV (7.5  $\mu$ M) with and without pre-treatment with DHBA (10  $\mu$ M). As an endogenous antioxidant, the GSH level in HPAEC was inversely related to the amount of oxidative stress in the cells. Overnight treatment of HPAEC with RTV (7.5  $\mu$ M) led to a reduction in glutathione (GSH) levels by 75% ( $P<0.05$ ), indicating increased oxygen tension. Meanwhile, pretreatment for one hour with dihydroxybenzyl alcohol at 10  $\mu$ M or 50  $\mu$ M increased the level of GSH in a dose-dependent manner by 50% and 76%, respectively, over the level seen with RTV treatment alone ( $P<0.05$ ).  $n=4$ ,  $P<0.05$ .

tension. Meanwhile, pretreatment for one hour with dihydroxybenzyl alcohol at 10  $\mu$ M or 50  $\mu$ M prior to RTV administration increased the level of GSH in a dose-dependent manner by 50% and 76%, respectively, over the level seen with RTV treatment only overnight ( $P<0.05$ ); these data indicate a dose-dependent reduction in oxidative stress (Figure 4).

#### DHBA reduces superoxide anion levels in porcine pulmonary arteries

One mediator of the deleterious effects of RTV in endothelial cells is the increased level of superoxide anion seen after treatment with RTV [7,18]. Dysregulation of the NOS system can lead to increased superoxide anion formation, and eNOS can actually become a producer of superoxide anion, a process known as eNOS uncoupling [19]. We implemented a luciferin chemiluminescence assay to determine superoxide anion levels in porcine pulmonary artery endothelium after treatment with RTV (15  $\mu$ M) with and without DHBA (10  $\mu$ M) compared to controls. Treatment with RTV did lead to a marked increase in superoxide anion levels as measured by relative light units/s/mm<sup>2</sup> (RLU/s/mm<sup>2</sup>), which increased by 79% in comparison with control vessels. However, when these rings were concomitantly treated with DHBA, there was a statistically significant decrease in ROS production, which was dose-dependent; co-treatment with RTV and DHBA (10  $\mu$ M) reduced ROS by 70% when compared to treatment with RTV alone ( $P<0.05$ ), while 50  $\mu$ M DHBA led to an 84% decrease ( $P<0.05$ ). There was



**Figure 5.** Porcine pulmonary artery superoxide assay. Using a lucigenin chemiluminescence assay, the superoxide level in porcine PA endothelium was measured after treatment with RTV (15 μM), DHBA (10 μM), or RTV and DHBA (10 or 50 μM). While RTV treatment increased the level of superoxide anion in the endothelium, co-treatment with DHBA reduced this effect in a dose-dependent manner. n=9, P<0.05.

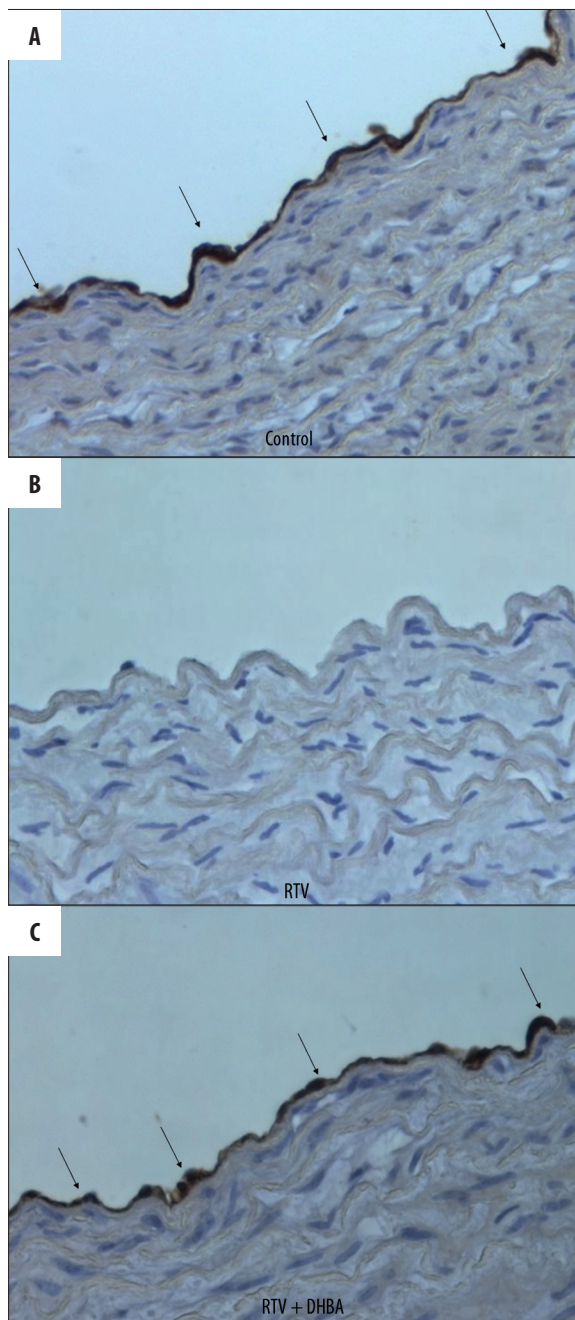
no significant difference in ROS production between rings treated with only DHBA (10 μM) and controls (Figure 5).

**DHBA restores endothelial Nitric Oxide Synthase expression in porcine pulmonary arteries**

Immunohistochemical staining was performed on porcine pulmonary artery rings after overnight treatment with RTV (15 μM) with or without DHBA (50 μM), or no treatment controls. While treatment with RTV reduced the expression of eNOS, co-treatment with DHBA at 50 μM restored eNOS expression histologically (Figure 6). It has previously been shown that RTV reduces eNOS expression in HPAEC and porcine pulmonary artery [7]. It is likely that changes in eNOS expression are involved in the endothelial dysfunction and oxidative stress seen in RTV-treated cells, which may be blocked by treatment with antioxidant DHBA.

**DISCUSSION**

In the current study, we demonstrate that DHBA has a powerful antioxidant effect in cell-free systems that surpasses that of vitamin C with regards to scavenging ability. Also, we used a well-characterized myograph model and a concentration of RTV near plasma levels in humans (15 μM) to demonstrate the ability of DHBA to reverse the deleterious effects of RTV on vasomotor dysfunction in porcine pulmonary arteries [20,21]. DHBA also ameliorates RTV-induced increases in ROS in HPAEC and porcine pulmonary arteries and restores eNOS staining in porcine pulmonary artery rings. These results indicate the potential benefit of this compound in counteracting the harmful effects of PI treatment for HIV on the vasculature, which appear to be mediated by ROS generation and eNOS down-regulation. Thus DHBA and DHBA-derived compounds may have potential



**Figure 6.** Effects of DHBA on the RTV-induced eNOS downregulation in porcine pulmonary artery rings. Fresh porcine pulmonary artery rings were incubated overnight after treatment with RTV (15 μM), RTV and DHBA (50 μM), or no-treatment control. Immunohistochemical analysis was performed. n=5 A. Porcine PA with no treatment. B. RTV treatment reduced eNOS staining. C. Treatment with DHBA restored staining of eNOS along the endothelium.

therapeutic use in the prevention and/or treatment of oxidative stress-mediated pulmonary artery endothelial dysfunction induced by HAART.

Oxidative stress in endothelial cells occurs when there is imbalance between endogenous oxidative enzymes and antioxidants, and it is an important contributor to vascular

disease. Endothelial NO produced by eNOS plays a major role in normal endothelial cellular homeostasis by regulating vascular tone and promoting an antiproliferative overall state. Meanwhile, in the presence of oxidative stress, eNOS becomes dysfunctional [17]. Elevated levels of ROS can also decrease NO bioavailability by reacting with NO to form peroxynitrite, which can lead to oxidation of the essential eNOS-cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH<sub>4</sub>), leading in turn to eNOS uncoupling and increased superoxide anion production [19].

Homeostasis with regards to the redox state of the cell is also maintained by the action of endogenous antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and others. It has been shown that in the lungs of patients with idiopathic PAH, levels of glutathione peroxidase and superoxide dismutase, important endogenous antioxidants, are reduced [22]. Meanwhile, gene transfer of superoxide dismutase into rats treated with monocrotaline to induce PAH led to a reduction in vascular remodeling and smooth muscle cell proliferation, as well as reduced right ventricular systolic pressure, a marker of PA pressure [23]. Clearly, oxidative stress contributes to the vascular lesions of PAH, and the reduction of endothelial reactive oxygen species represents an important potential mechanism for prevention and/or therapy of this condition.

Based on DHBA's superoxide scavenging activity and DPPH scavenging activity in a cell free system, DHBA has powerful antioxidant properties that compare favorably with those of Vitamin C. We also found that treatment with DHBA does, indeed, reduce endothelial dysfunction (as evidenced by reduced endothelium-dependent relaxation) that is induced by RTV treatment in porcine pulmonary arteries. DHBA also blocks superoxide anion production in porcine pulmonary arteries treated with RTV and restores eNOS expression based on immunohistochemical staining. Finally, DHBA lowers the level of oxidative stress in HPAEC treated with RTV, as evidenced by lower GSH levels. This is in accordance with a large clinical study that increasing the phenolic content of olive oil in the diet reduces oxidative stress as evidenced by improved glutathione antioxidant status [24].

In a randomized trial, a Mediterranean diet supplemented with olive oil or mixed nuts led to a reduced blood pressure and lipid profiles, decreased insulin resistance, and reduced serum inflammatory markers when compared to a lowfat diet [25]. While the healthful benefits of virgin olive oil consumption have typically been considered to be due to the high concentration of monounsaturated fatty acids, the phenolic compounds in virgin olive oil have also been found to improve physiologic parameters. This has been demonstrated in a variety of clinical settings and includes a reduction in inflammatory biomarkers like IL-6 and CRP, reduced LDL oxidation, increased HDL level, and increased endogenous antioxidant activity (reviewed in [26]). As such, the phenolic fraction of olive oil is known to possess powerful antioxidant properties [26,27]. When in a compound mixture, the phenols in olive oil have been shown to reduce the lipid peroxidation that leads to tissue damage in rats treated with ferric-nitrosyltriacetate, a powerful oxidating agent [28]. The phenolic fraction of olive oil has also been shown to protect human red blood cells (RBC) from oxidative hemolysis [13]. DHBA is found in the phenolic

fraction of olive oil, and we demonstrated that it has antioxidant properties in a cell free system and in pulmonary artery endothelial cells. While this compound has not been studied in humans or animals, it represents a promising therapy for the promotion of endothelial health.

## CONCLUSIONS

In patients infected with HIV, PAH-HIV remains a life-threatening complication [29]. The endothelium appears to be the primary culprit in PAH [4,5], and most current therapies are aimed at restoration of endothelial health [30,31]. We have previously shown that RTV causes endothelial dysfunction in porcine pulmonary arteries and HPAEC, which appears to be due to an increase in oxidative stress and reduction in eNOS expression [7]. In this investigation, we demonstrated that DHBA, a small molecule found in the phenolic fraction of virgin olive oil, functions as an antioxidant in porcine pulmonary arteries and HPAEC, reducing the deleterious effects of RTV mediated by oxidative stress. The molecular mechanism whereby this small molecule exerts its vasoprotective role in endothelial cells remains to be determined. Meanwhile, DHBA may represent an important therapeutic tool in the treatment and/or prevention of RTV-induced PAH in patients with HIV.

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