Antioxidant treatment attenuates pulmonary arterial hypertension-induced heart failure

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1Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center Amsterdam, Amsterdam; 2Image Sciences Institute and 3Department of Cardiology, Division of Heart and Lungs, University Medical Center Utrecht, Utrecht; and 4Department of Anesthesiology, VU University Medical Center Amsterdam, Amsterdam, The Netherlands

Submitted 28 January 2009; accepted in final form 7 January 2010


Redout EM, van der Toorn A, Zuidwijk MJ, van de Kolk CW, van Echteld CJ, Musters RJ, van Hardeveld C, Paulus WJ, Simonides WS. Antioxidant treatment attenuates pulmonary arterial hypertension-induced heart failure. Am J Physiol Heart Circ Physiol 298: H1038–H1047, 2010. First published January 8, 2010; doi:10.1152/ajpheart.00097.2009.—ROS have been implicated in the development of pathological ventricular hypertrophy and the ensuing contractile dysfunction. Using the rat monocrotaline (MCT) model of pulmonary arterial hypertension (PAH), we recently reported oxidative stress in the failing right ventricle (RV) with no such stress in the left ventricle of the same hearts. We used the antioxidant EUK-134 to assess the role of ROS in the pathological remodeling and dysfunction of the RV. PAH was induced by an injection of MCT (80 mg/kg, day 0), treatment with EUK-134 (25 mg/kg, once every 2 days) of control and MCT-injected animals [congestive heart failure (CHF) group] was started on day 10, and animals were analyzed on day 22. EUK-134 treatment of the CHF group attenuated cardiomyocyte hypertrophy and associated changes in mRNA expression (myosin heavy chain and deiodinase type 3). It also reduced RV oxidative stress and proapoptotic signaling and prevented interstitial fibrosis. Cardiac MRI showed that ROS scavenging did not affect the 37% increase in end-diastolic volume of the RV in the CHF relative to the control group, but the threefold increase in end-systolic volume was reduced by 42% in the EUK-134-treated CHF group. The improved systolic function was confirmed using echocardiography by an assessment of tricuspid annular plane systolic excursion. These changes were specific to the overloaded RV, since they were not observed in the left ventricles (LVs) of the same hearts (28). The oxidative stress may at least in part account for the activation of proapoptotic pathways found in the failing RV in PAH (2) but may also account for other aspects of pathological remodeling. An in vitro study (33) has shown that ROS may act as signaling molecules driving cardiomyocyte hypertrophy. A study (34) of LV remodeling has shown that high cellular ROS levels activate matrix remodeling and increase fibrosis, which are also aspects of PAH-induced RV hypertrophy. In addition, high ROS levels may lead to contractile dysfunction by directly affecting proteins involved in Ca2+ handling and contraction (10, 41). A study (34) of pathological LV remodeling in which the antioxidant capacity of myocytes was modulated pharmacologically or genetically generally also pointed to a role of ROS in this process.

Taken together, the available data suggest the involvement of oxidative stress in PAH-induced RV remodeling and dysfunction, but the possibility of a causal role for ROS in this process has so far not been investigated. This is a particularly relevant issue since the treatment options for PAH patients are limited and increased RV ROS activity may be amenable to therapeutic intervention aimed at alleviating the principal secondary problem of PAH. Therefore, in the present study, we used the synthetic antioxidant EUK-134 to test the hypothesis that increased ROS production is a causative factor in the development of PAH-induced pathological remodeling of the RV. EUK-134 belongs to a class of salen-manganese complexes and is a superoxide dismutase and catalase mimetic (7) capable of scavenging ROS of both cytosolic (29) and mitochondrial origin (24).

As in our previous studies (2, 28), we used the monocrotaline (MCT)-treated rat as an experimental model of PAH-induced RV failure. MCT is a pyrrolizidine alkaloid, and its bioactive metabolite selectively injures the vascular endothelium of lung vessels. Progressive pulmonary vasculitis leads to increasing vascular resistance and a gradual rise in arterial pressure starting ~7 days after a single dose of MCT (11). The increase in RV afterload induces hypertrophy, which, depending on the dose of MCT used, progresses to dilation and failure (2, 11, 14). Our data now show that treatment with EUK-134 reduces oxidative stress, attenuates pathological remodeling, and improves contractile function in PAH-induced RV hypertrophy.

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EUK-134 ATTENUATES RV FAILURE

MATERIALS AND METHODS

Animals. Animals were treated according to national guidelines and with permission of the Institutional Animal Care and Use Committee of the Vrije University Medical Center Amsterdam (Amsterdam, The Netherlands), which conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Male Wistar rats (Harlan) were housed individually (250 cm²/animal) and received food and water ad libitum. Rats were randomized and received a single subcutaneous injection with either saline (control group) or 80 mg MCT/kg body wt [congestive heart failure (CHF) group]. Animals were killed 22 days after MCT treatment with an isoﬂurane overdose, and their heart and lungs were excised. Lungs were weighed as a measure of the degree of pulmonary vasculitis and as an indication of the increase in pulmonary arterial pressure (2). Transmural biopsies (78.54 mm²) of the RV free wall were weighed to determine wall thickness. Ventricular tissue samples for immunoblot analysis and RNA extraction were snap frozen in liquid nitrogen and stored at −80°C.

Antioxidant treatment. Animals were randomized to treatment with either EUK-134 (Cayman Chemical, 25 mg/kg in PBS) by an intraperitoneal injection or an equal volume of vehicle. Animals were injected five times over the course of 10 days, starting at day 10 after MCT treatment. Animals were killed on day 22 after MCT treatment.

Cardiac MRI procedures. Cardiac MRI was performed immediately before animal euthanization using a 9.4-T horizontal bore magnetic resonance system equipped with a 12-cm-inner diameter gradient set with gradients up to 500 mT/m (Varian, Palo Alto, CA) and a quadrature volume coil with an inner diameter of 72 mm (Rapid Biomedical, Rimpar, Germany). For detailed information, see the online Supplemental Material.1

Image analyses. Images were analyzed with Segment version 1.65 (http://segment.heidelberg.se). The phases corresponding to the largest (end diastolic) and smallest (end systolic) RV volumes were used to measure the septal-lateral end-diastolic diameter (EDD) and end-systolic diameter (ESD) and to calculate stroke volume (SV) and ejection fraction (EF). The product of SV and heart rate was expressed as RV cardiac output (CO).

Echocardiography. After cardiac MRI, noninvasive echocardiographic measurements were performed with a Hewlett-Packard Sonos 5500 instrument (Hewlett-Packard) using a 15-MHz transducer (15-6L linear probe, Philips) applied parasternally to the shaved chest wall of rats anesthetized with 1.5% isoflurane in a mixture of air and nitrous oxide (2). Transmural biopsies (78.54 mm²) of the RV free wall were weighed to determine wall thickness. Ventricular tissue samples for immunoblot analysis and RNA extraction were snap frozen in liquid nitrogen and stored at −80°C.

CARDIAC FUNCTION AND HEMATOXYLIN AND EOSIN-STAINED SECTIONS

Hematoxylin and eosin-stained sections by quantitative image analysis was determined. Ventricular tissue samples were excised. Lungs were weighed as a measure of the degree of pulmonary vasculitis and as an indication of the increase in pulmonary arterial pressure (2). Transmural biopsies (78.54 mm²) of the RV free wall were weighed to determine wall thickness.

RESULTS

Previously, we (2) showed that distinct gene expression profiles predictive of failure were already present 14 days after the injection of MCT (80 mg/kg, CHF group). Because of the potential role of ROS in early hypertrophic signaling as well as in ultimate contractile dysfunction, EUK-134 treatment of the CHF and control groups was started on day 10 after the injection of MCT or carrier, respectively. Treatment with EUK-134 was continued until the time of death on day 22.

General features. A previous study (2) has shown that body weight gain is reduced in MCT-treated animals, resulting in 10% lower body weight on day 18 compared with controls. Those animals that develop CHF then start to lose weight (2). In line with this, the data shown in Table 1 demonstrate a 15% lower body weight of MCT-treated groups on day 22 compared with the control group, indicative of the onset of CHF. RV hypertrophy was indicated by a 35% increase in RV wall thickness. Treatment with EUK-134 did not affect these parameters, nor did it affect lung wet weight in the control + EUK-134 and CHF + EUK-134 groups (Table 1). The increase in lung wet weight after MCT treatment reflects pulmonary vasculitis and is not related to edema, since the wet weight-to-dry weight ratios did not differ between groups (Table 1). Analysis of an echocardiographic index of PAH, i.e., PAA/T (12, 13), showed that the degree of PAH was indeed the same in the CHF and CHF + EUK-134 groups (Table 1). This was furthermore supported by the equally high expression level in the CHF and CHF + EUK-134 groups of atrial natriuretic factor mRNA in the RV (Table 1), which was shown earlier to increase in proportion to the degree RV hypertrophy (2, 23), which relates to the degree of PAH.

EUK-134 treatment attenuates RV dysfunction. The cardiac MRI images shown in Fig. 1 of midventricular slices at systole and diastole demonstrate the remodeling of the RV induced by PAH (CHF group). LV geometry is consequently affected, with septal flattening in diastole and inward movement in acid (TF(A; pH 7.2). Homogenates were centrifuged at 12,000 g for 10 min, and the pellet was resuspended in 200 μl buffer containing 6% SDS, 0.01% TFA, and 50 mM DTT and assayed for protein concentration. The Oxyblot Oxidized Protein Detection kit was purchased from Chemicon. Protein (15 μg) was derivatized with 1,3-dinitrophenylhydrazine (DNPH) for 15 min according to the manufacturer’s instructions and followed by one-dimensional electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and then stained with Ponceau red. After an incubation with anti-dinitrophenyl antibody, blots were developed using a chemiluminescence detection system. Bands were visualized by ECL-Advanced (Amersham) and quantified using a FujiFilm LAS 3000 laser densitometer. To quantify the level of protein oxidation, we calculated the ratio between densitometric values of the Oxyblot bands and those stained with Ponceau red (3, 5).

Real-time PCR. Quantitative real-time PCR was performed using the Applied Biosystems model 7700 using standard cycle parameters in combination with SYBR green (Applied Biosystems) as previously described (2). For the primer sequences used in this study, see the online Supplemental Material.

Statistical analysis. Results are presented as means ± SE. Data were analyzed using Student’s t-test or one-way ANOVA with the Bonferroni correction for multiple testing. P values of <0.05 were considered significant.

1 Supplemental Material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.
Data are expressed as means ± SE; n, number of animals. The general characteristics of control, congestive heart failure (CHF), and EUK-134-treated animals are shown. Right ventricular (RV) free wall thickness was calculated from the weight of a transmural biopsy with a surface area of 78.54 mm². Pulmonary artery acceleration time normalized to cycle length (PAAT/CL) was determined by echocardiography as an index of pulmonary arterial hypertension (12,13). The expression of atrial natriuretic factor (ANF) mRNA was determined by RT-PCR and normalized to the expression of HPRT (see MATERIALS AND METHODS for details). Heart rate did not differ significantly between the experimental groups and averaged 350 beats/min. *P < 0.05 vs. the control group; †P < 0.05 vs. the control + EUK-134 group.

**Table 1. General characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Group</th>
<th>Control + EUK-134 Group</th>
<th>CHF Group</th>
<th>CHF + EUK-134 Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Body weight, g</td>
<td>305 ± 6</td>
<td>299 ± 6</td>
<td>260 ± 5**†</td>
<td>251 ± 9**†</td>
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<tr>
<td>Tibia length, cm</td>
<td>3.76 ± 0.02</td>
<td>3.73 ± 0.03</td>
<td>3.77 ± 0.03</td>
<td>3.66 ± 0.04</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>867 ± 18</td>
<td>897 ± 31</td>
<td>951 ± 20</td>
<td>876 ± 9</td>
</tr>
<tr>
<td>RV weight, mg</td>
<td>149 ± 21</td>
<td>117 ± 6</td>
<td>281 ± 10†</td>
<td>298 ± 23†</td>
</tr>
<tr>
<td>RV-to-heart weight ratio</td>
<td>0.17 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.30 ± 0.01†</td>
<td>0.34 ± 0.03†</td>
</tr>
<tr>
<td>Wall thickness, mm</td>
<td>1.24 ± 0.04</td>
<td>1.21 ± 0.06</td>
<td>1.67 ± 0.06†</td>
<td>1.70 ± 0.07†</td>
</tr>
<tr>
<td>Lung wet weight, g</td>
<td>1.06 ± 0.09</td>
<td>1.25 ± 0.03</td>
<td>2.21 ± 0.13†</td>
<td>1.95 ± 0.12†</td>
</tr>
<tr>
<td>Lung wet-to-dry weight ratio</td>
<td>4.81 ± 0.07</td>
<td>4.91 ± 0.19</td>
<td>4.58 ± 0.27</td>
<td>4.36 ± 0.43</td>
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<tr>
<td>PAAT/CL</td>
<td>0.152 ± 0.016</td>
<td>0.178 ± 0.017</td>
<td>0.060 ± 0.006†</td>
<td>0.081 ± 0.015†</td>
</tr>
<tr>
<td>RV ANF mRNA expression, arbitrary units</td>
<td>1.00 ± 0.94</td>
<td>0.54 ± 0.49</td>
<td>5.48 ± 0.72†</td>
<td>5.73 ± 1.93†</td>
</tr>
</tbody>
</table>

**Systole. Two sets of images are shown for the CHF + EUK-134 group showing the range of the effect of EUK-134 treatment observed in this study. Figure 2 shows EDD and ESD of the RV as well as the RV volumes at diastole and systole obtained from the cardiac MRI analyses. EDV was increased by 37%, whereas ESV showed a threefold increase, corresponding to a fivefold increase in ESD. EUK-134 treatment of CHF animals from day 10 onward did not affect diastolic dimensions but resulted in a significantly lower ESD (~28%) and a concomitant 42% reduction of ESV compared with the control group (Fig. 2). Treatment of control animals did not affect any of the parameters. The attenuating effect of EUK-134 treatment of CHF animals on changes in RV SV, EF, and CO is shown in Fig. 3, A–C, respectively. In addition, TAPSE was determined by echocardiography as a further index of RV systolic function (Fig. 3D). In line with the positive effect of EUK-134 treatment on EF, the decrease in TAPSE seen in CHF animals was significantly less in the CHF + EUK-134 group.**

**EUK-134 treatment reduces oxidative stress in the RV myocardium.** To assess the effect of EUK-134 treatment on oxidative stress, we stained RV tissue slices for nitrotyrosine and analyzed tissue homogenates for protein oxidation using the Oxyblot technique (3, 5). The images shown in Fig. 4A demonstrate extensive nitrotyrosine staining in CHF, confirming our previous results (28). Markedly less staining was observed in the RV of CHF + EUK-134-treated animals. Similarly, the RV myocardium from CHF animals showed increased levels of protein oxidation compared with controls, whereas this increase was markedly attenuated by EUK-134 treatment (Fig. 4B). This is corroborated by the effect of EUK-134 treatment on the mRNA expression level of mitochondrial (Mfn)-2. Mfn-2 is a mitochondrial membrane protein that can trigger cardiomyocyte apoptosis, and its expression has been recently shown to be upregulated by oxidative stress (31). The high RV Mfn-2 mRNA expression seen in the CHF group was virtually absent in the CHF + EUK-134 group (Fig. 4C). Oxidative stress and proapoptotic signaling in PAH-induced RV remodeling has previously been shown to be also associated with increased mRNA expression of the cell cycle regulator cyclin D1 (2, 8). The results shown in Fig. 4D demonstrate that EUK-134 treatment also attenuated this effect of PAH.

**Effect of EUK-134 on cardiomyocyte hypertrophy and fibrosis.** Hematoxylin and eosin stainings were performed to determine the average cardiomyocyte CSA in RV sections. Representative images and the results of the quantitative analyses are shown in Fig. 5, A and B, respectively, and demonstrate significantly less concentric hypertrophy of the cardiomyocytes in the CHF + EUK-134 group relative to the CHF group. Upregulation of the mRNA level of myosin heavy chain (MHC)-β, a characteristic feature of pathological cardiomyocyte hypertrophy, was also significantly attenuated by EUK-134 treatment (Fig. 5C). Similarly, expression of mRNA of the thyroid hormone-inactivating enzyme deiodinase type 3 (D3), which has also been shown to be upregulated in RV myocyte hypertrophy (32, 40), was prevented by EUK-134 treatment of the CHF group (Fig. 5D). Collagen content was next quantified...
after the staining of RV sections with Picrosirius red. The results shown in Fig. 6, A and B, demonstrate that EUK-134 treatment prevented the interstitial fibrosis that characterizes RV tissue of the CHF group.

**DISCUSSION**

In the present study, we show in a model of PAH-induced RV hypertrophy that treatment with the antioxidant EUK-134 attenuates the development of cardiomyocyte hypertrophy, decreases RV oxidative stress, and prevents RV fibrosis. The overall effect of ROS scavenging on pathological remodeling of the RV results in significantly improved systolic function.

Effect of EUK-134 treatment on PAH-induced RV remodeling and dysfunction. A single dose of MCT of 60 or 80 mg/kg has been previously shown to induce progressive PAH,
resulting in RV hypertrophy and, ultimately, failure (13, 14). Assessment of PAH by echocardiography (13) or radiotelemetry (11) shows that PAH starts to develop 1 wk after MCT administration. RV hypertrophy is evident at 2 wk, reaching a 45–75% increase in wall thickness around the fourth week (2, 13, 21), with progressive dilation and diminished EF and CO (11, 14, 35). Most animals become cachexic after the third week and succumb before the sixth week with signs and symptoms of CHF (2, 13, 14, 21). A time-course study (13) has shown that after the onset of RV dilation, EDV increases more than twofold over the course of 10 days, culminating in overt failure. Our present data

Fig. 4. A: representative nitrotyrosine-stained RV sections from the CON, CON+EUK, CHF, and CHF+EUK groups. Green, blue, and red colors represent nitrotyrosine, nuclei, and sarcolemma, respectively. B: detection of oxidized protein in RV homogenates by the Oxyblot procedure (see MATERIALS AND METHODS for details). C and D: expression of mRNA of mitofusin (Mfn)-2 (C) and cyclin D1 (D) was determined in RV tissue by RT-PCR and normalized to the expression of HPRT (see MATERIALS AND METHODS for details). The expression levels of Mfn-2 mRNA in the CON and CON+EUK groups were below the detection limit. Values are expressed as means ± SE; n = 6 for the CON group, 5 for the CON+EUK group, 5 for the CHF group, and 5 for the CHF+EUK group. *P < 0.05 vs. the CON group; #P < 0.05 vs. the CON+EUK group; $P < 0.05 vs. the CHF group.
Fig. 5. A: representative hematoxylin and eosin-stained RV sections of CON, CON+EUK, CHF, and CHF+EUK animals. B: quantification of cardiomyocyte cross-sectional area (CSA) from the indicated groups (see MATERIALS AND METHODS for details). Values are expressed as means ± SE; n = 4 for the CON group, 6 for the CON+EUK group, 6 for the CHF group, and 6 for the CHF+EUK group. *P < 0.05 vs. the CON group; #P < 0.05 vs. the CON+EUK group; $P < 0.05 vs. the CHF group. The average cardiomyocyte CSA in the LV did not differ between groups (data not shown).

C and D: gene expression of myosin heavy chain (MHC)-β (C) and deiodinase type 3 (D3; D) was determined in RV tissue from all experimental groups by RT-PCR and normalized to the expression of HPRT (see MATERIALS AND METHODS for details). Values are expressed as means ± SE; n = 6 for the CON group, 5 for the CON+EUK group, 5 for the CHF group, and 5 for the CHF+EUK group. *P < 0.05 vs. the CON group; #P < 0.05 vs. the CON+EUK group; $P < 0.05 vs. the CHF group.
obtained on day 22 after MCT administration confirm these previous studies, showing a 35% increase in RV wall thickness (11, 13, 14) and a moderate degree of RV dilation, i.e., a 37% increase in EDV (Fig. 2) (13). Altered ventricular geometry was evident, particularly with respect to the shape of the septum during the cardiac cycle (Fig. 1). Septal flattening during diastole and bulging into the LV cavity during systole has also been described for PAH-induced RV hypertrophy and failure in patients, where it is was shown to affect LV filling (22).

The data shown in Fig. 3, A and B, demonstrate a 35% reduction in SV due to a 54% drop in EF in the CHF group. The systolic dysfunction was corroborated by a similar decrease in the echocardiographic parameter TAPSE (Fig. 3D). This degree of systolic dysfunction has been previously found at the time of overt failure [day 28 after MCT administration (80 mg/kg)] using either echocardiography (11) or pressure-volume measurements (14, 35). In these studies, RV dilation had progressed to a two- to threefold increase of EDV. Our data on day 22 now show that systolic dysfunction is already near maximal when RV dilation is still moderate.

EUK-134 treatment from day 10 to day 22 significantly attenuated the decline in systolic function induced by PAH, with EF and TAPSE both being 55% higher in the CHF + EUK-134 group compared with the CHF group (Fig. 3). Different indicators of the degree of PAH, i.e., PAAT/CL (12, 13), the lung wet weight-to-dry weight ratio, and, indirectly, the expression level of atrial natriuretic factor mRNA in the RV (2, 23), were the same in the treated and untreated groups (Table 1). Furthermore, the RV wall thickness and diastolic dimensions in the CHF and CHF + EUK-134 groups are indicative of the same degree of RV afterload. These data do not suggest a substantial effect of EUK-134 on the remodeling of the pulmonary vasculature, and hence on the degree of PAH, between days 10 and 22 after MCT administration. However, since we did not examine the pulmonary vasculature in this study, we cannot exclude that EUK-134 attenuated the progression of vascular remodeling to some degree. In this respect,
it is worth noting that using the rat MCT model, Csiszar et al. (4) recently reported that Resveratrol, an antioxidant with anti-inflammatory and endothelium-protective effects, prevented the remodeling of pulmonary arteries when given daily from the first day after MCT administration.

None of the parameters examined in our study were affected by EUK-134 treatment of the control group, and, taken together, our data suggest a predominantly direct action of EUK-134 on the overloaded cardiomyocyte, improving contractile properties in the treated group. Analyses of isolated hearts (18) as well as muscle strips (21), RV trabeculae (17, 28), and papillary muscles (39) have shown that MCT-induced hypertrophy results in a reduction of the rate of force development and force decline, a reduction of maximal force, and a flat or even negative force-frequency relation. A shift in contractile protein expression from the fast MHC-α isoform to the slow MHC-β isoform underlies part of these changes (16, 17). This shift is a hallmark of pathological ventricular hypertrophy, and in the MCT model it becomes evident at both the mRNA and protein level 2 wk after MCT administration, i.e., at the onset of hypertrophy (17, 38). The data on myocyte CSA as well as on MHC-β mRNA expression shown in Fig. 5, B and C, demonstrate that EUK-134 treatment reduces concentric myocyte hypertrophy and prevents at least part of the associated changes in contractile gene expression. The latter effect provides an explanation for the observed partial preservation of systolic function in the CHF + EUK-134 group. We (32) recently showed in this model that RV myocyte hypertrophy and concomitant cellular hypoxia stimulates the expression of thyroid hormone-degrading enzyme D3, possibly as part of an adaptive mechanism aimed at reducing cellular metabolism (32). EUK-134 treatment fully prevented the stimulation of D3 mRNA expression (Fig. 5D), suggesting that the reduction of myocyte hypertrophy is sufficient to prevent cellular hypoxia.

Effect of EUK-134 treatment on PAH-induced ROS production and action in the RV. EUK-134 is a ROS scavenger belonging to a class of superoxide dismutase and catalase mimetics capable of dismutating O$_2^-$ to H$_2$O$_2$, with further catalytic breakdown to O$_2$ and H$_2$O, and of breaking down nitrosative species (30). The effect of EUK-134 on myocyte hypertrophy, despite the same degree of PAH, corroborates previous studies (25, 34) in which ROS were shown to be the downstream effector of various hypertrophic stimuli, both in vitro and in vivo. Mechanical stretch of isolated cardiomyocytes was shown to increase cellular ROS production, and the concomitant hypertrophy could be prevented by superoxide dismutase/ catalase mimetics, including EUK-8, which is comparable to EUK-134 (7, 26). Treatment with the antioxidants N-2-mercaptopropionylglycine (6) and N-acetylcysteine (15) was shown to diminish LV hypertrophy in mice subjected to pressure overload. More recently, EUK-8 treatment of mice subjected to transaortic constriction (TAC) for a period of 28 days prevented cardiomyocyte hypertrophy and improved systolic function (37). Potential sources of increased cardiomyocyte ROS production include NADPH and xanthine oxidase activities, the mitochondrial electron transport chain, and uncoupling of nitric oxide synthase 3 (34). Of these, NADPH oxidases play a major role in cardiac redox signaling and are thought to relay hypertrophic stimuli, including pressure overload (25). Previously, we (28) found increased ROS-producing activity from NADPH oxidase in RV failure. Mitochondria were an additional source of ROS at this stage, but this activity becomes particularly important when myocyte hypertrophy has progressed and cellular hypoxia ensues (28). EUK-134 treatment diminished hypertrophy and cellular hypoxia, as mentioned above, and this source of ROS may therefore be expected to be reduced. Together with the powerful mitochondrial and cytosolic ROS-scavenging activity of EUK-134 (24, 29), this resulted in markedly less oxidative stress, as indicated by the reduction of protein carbonylation and nitration damage seen in the CHF + EUK-134 group (Fig. 4, A and B). The level of nitrotyrosine staining in the CHF group on day 22 was comparable with what we found earlier on day 28 (28), and the attenuating effect of EUK-134 on protein oxidation will contribute to the preservation of contractile function, given the inhibitory effect of ROS-induced damage of proteins involved in Ca$^{2+}$ regulation and energy metabolism, among others (10, 41).

High levels of ROS have been shown to be associated with the activation of apoptosis and fibrosis in LV hypertrophy (34). In the previously mentioned study (34) of LV hypertrophy in TAC mice, treatment with EUK-8 prevented fibrosis and cardiomyocyte apoptosis, particularly in a mutant mouse with reduced endogenous capacity for scavenging of mitochondrial free radicals. Although fibrosis is not consistently observed in the MCT-model (14), we found significant interstitial fibrosis in the hypertrophic RV, in agreement with other studies (9, 16, 21). Furthermore, the observed effect of EUK-134 treatment on the level of fibrosis (Fig. 6) indicates that matrix remodeling is also in the overloaded RV at least in part dependent on increased ROS levels, confirming the earlier proposed role of ROS-dependent activation of matrix metalloproteinases in PAH-induced RV remodeling (35). Similarly, our data confirm the suggested role of ROS in the activation of proapoptotic pathways in the hypertrophic RV (2, 8, 28). ROS are known to activate such pathways in cardiomyocytes (1), and their up-regulation together with downregulation of antiapoptotic Bcl-2 has been shown in PAH-induced RV failure (2, 8) and also shown to be associated with cardiomyocyte apoptosis and the upregulation of cyclin D1 (8). The observed increased levels of Mnf-2 mRNA (Fig. 4C) corroborated a role of ROS, since Mnf-2 expression is stimulated by oxidative stress and triggers cardiomyocyte apoptosis through the activation of the mitochondrial cell death pathway (31). Finally, EUK-134 treatment largely prevented the upregulation of Mnf-2 and cyclin D1 expression in the CHF group, indicating a causal relationship between ROS and cardiomyocyte apoptosis. Although we did not quantify apoptosis, our morphological data suggest that EUK-134 treatment may indeed have prevented myocyte loss in the hypertrophic RV. RV wall thickness was increased by ~37% in the CHF and CHF + EUK-134 groups relative to control groups (Table 1), and end-diastolic dimensions were also similarly increased in both groups (Fig. 2). In line with the degree of hypertrophy, the average cardiomyocyte CSA was increased by ~32% in the CHF + EUK-134 group, whereas the CSA was significantly higher in the untreated group (Fig. 5), indicative of a lower number of cardiomyocytes in the RV of the CHF group compared with the EUK-134-treated group.

Clinical perspectives. Current treatment options of PAH are largely targeted at reducing pathological vascular remodeling. Although improvement of clinically relevant end points is achieved with various pharmacological strategies, there is no...
cure for PAH, and RV failure is often the fatal complication as the disease progresses. The present study shows, for the first time, a critical role for oxidative stress in the onset of RV hypertrophy and the subsequent development of contractile dysfunction in a model of PAH. With the current dose of EUK-134 of 25 mg/kg given every 2 days, scavenging of cardiomyocyte ROS diminished myocardial fibrosis, and possibly apoptosis and largely preserved systolic function in the pressure-overloaded RV. The present data show the potential benefit of the EUK class of salen-manganese complexes as a complementary therapy in the treatment of PAH, particularly in the early stages of the disease.

ACKNOWLEDGEMENTS

The authors thank Dr. Leon de Windt for the helpful suggestions concerning the design of the study. The authors also thank Nicky Boontje for optimizing the Oxyblot procedures.

GRANTS

Part of the experiments were performed on a magnetic resonance scanner funded by The Netherlands Organization for Scientific Research.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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17. Oxyblot procedures.


