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Severe Vitamin E deficiency exacerbates acute hyperoxic lung injury associated with increased oxidative stress and inflammation

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Abstract

Hyperoxia causes acute lung injury along with an increase of oxidative stress and inflammation. It was hypothesized that vitamin E deficiency might exacerbate acute hyperoxic lung injury. This study used α-tocopherol transfer protein knockout (α-TTP KO) mice fed a vitamin E-deficient diet (KO E(-) mice) as a model of severe vitamin E deficiency. Compared with wild-type (WT) mice, KO E(-) mice showed a significantly lower survival rate during hyperoxia. After 72 h of hyperoxia, KO E(-) mice had more severe histologic lung damage and higher values of the total cell count and the protein content of bronchoalveolar lavage fluid (BALF) than WT mice. IL-6 mRNA expression in lung tissue and the levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in both lungs and BALF were higher in KO E(-) mice than in WT mice. It was concluded that severe vitamin E deficiency exacerbates acute hyperoxic lung injury associated with increased oxidative stress or inflammation.

Keywords: *Hyperoxia, vitamin E, oxidative stress, inflammation, α-tocopherol transfer protein*

Introduction

In vivo hyperoxia is known to cause pulmonary injury and respiratory failure that can be fatal [1]. The intracellular production of reactive oxygen species (ROS) during exposure to hyperoxia is widely held to be responsible for both lung injury *in vivo* and the death of cultured cells *in vitro* [2–5]. At the cellular level, the exposure of cultured pulmonary cells to hyperoxia activates signalling molecules related to programmed cell death mediated by intracellular ROS production [6]. *In vivo* hyperoxia can also trigger an inflammatory response in animal lungs. Hyperoxia causes pulmonary cells to increase the secretion of chemoattractants and other proinflammatory cytokines that promote leukocyte recruitment to the lungs. The leukocytes that are recruited in this way, including neutrophils and monocytes, accumulate within the pulmonary circulation, interstitium and air ways. After recruitment to the lungs, these cells are potent sources of additional ROS that can directly damage the lung tissue [7–10]. In recent studies using animal models of hyperoxia, overexpression of antioxidant enzymes has been shown to decrease the recruitment of inflammatory cells into the alveolar spaces and reduce proinflammatory chemokine/adhesion molecule expression as well as

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ROS production [11,12]. Likewise, the inhibition of inflammatory cell recruitment to the lungs by hyperoxia also reduces ROS production in newborn rats [13]. Therefore, the interaction between ROS and inflammation seems to establish a vicious cycle that initiates and/or exacerbates lung injury.

Vitamin E is a lipid-soluble, chain-breaking antioxidant that prevents the peroxidation of lipids in cell membranes and lipoproteins [14,15]. α-Tocopherol (α-T) is the most abundant and biologically active form of vitamin E [16] and α-T is found at high levels in human plasma. This is because α-tocopherol transfer protein (α-TTP), a cytosolic protein mainly expressed in the liver, selectively transfers α-T from lipoproteins taken up by hepatocytes to newly secreted lipoproteins and consequently determines the plasma α-T level [17,18]. α-TTP knockout (α-TTP KO) mice exhibits extremely low or undetectable levels of α-T in the plasma and tissues [19]. These mice have a normal appearance and growth at a young age [19], but exhibit increased basal levels of oxidative stress [20,21], suggesting that this model could be vulnerable to an additional oxidative insult.

Besides its antioxidant effect, α-T also shows anti-inflammatory activity that might be mediated via a non-antioxidant mechanism. Recent studies using activated monocytes have shown that α-T inhibits proinflammatory cytokine release [22,23] and decreases monocyte-endothelial cell adhesion [24], with these effects being unrelated to its antioxidant activity.

Vitamin E is known to be secreted together with alveolar surfactant by type II alveolar pneumocytes [25] and is thought to be distributed to the alveolar epithelium, so vitamin E is thought to protect the lungs as first line of defense against various inhaled materials. Indeed, in animal models of lung injury induced by oxidative/proinflammatory inhalants (LPS, cigarette smoke and bleomycin), vitamin E significantly reduces lung damage as well as oxidative stress and/or inflammation [26–29].

Taken together, these findings allow us to speculate that α-T administration could be beneficial for hyperoxic lung injury. However, few studies have assessed the effect of α-T on hyperoxic lung injury and their results have been unclear or conflicting [30–32]. Moreover, despite several investigations into the effect of vitamin E deficiency on animal lungs during hyperoxia [33,34], only limited information has been obtained. In the present study, we created a model of severe vitamin E deficiency by using α-TTP KO mice fed a vitamin E-deficient diet and then we examined the effect of severe vitamin E deficiency on lung injury, oxidative stress, inflammation and mortality after exposure to hyperoxia.

Materials and methods

Animals

Specific-pathogen-free adult male α-TTP$^{-/-}$ mice (B6.129S7 α-Ttp^{tm1Csk}) [19] were obtained from Chugai Pharmaceutical Technology Laboratory at 3–4 months of age. Their genotype was confirmed by PCR of DNA obtained from tail tissue. As a control, age-matched C57BL6J mice were purchased from Clea Japan Co. (Tokyo, Japan). Both strains of mice were divided into the following four groups: WT group, C57BL/6 mice fed a control diet (containing 10 mg of d-α-tocopheryl acetate/100g); WT E(-) group, C57BL/6 mice fed a vitamin E-free diet (< 0.1 mg of d-α-tocopheryl acetate/100g); KO group, α-TTP$^{-/-}$ mice fed a control diet; and KO E(-) group, α-TTP$^{-/-}$ mice fed a vitamin E-deficient diet. These two diets were based on a purified, synthetic diet providing all the essential nutrients to support laboratory rodents. The vitamin E-free diet was the basal diet after removal of α-tocopherol. The control diet was obtained by addition of d-α-tocopheryl acetate to the vitamin E-free diet (Table I). These diets were continued for a month until the next experiment or until measurements were performed. All diets were purchased from Clea Japan Co. (Tokyo, Japan). The α-T levels in the plasma and lung tissue were measured as reported later. Then WT and KO E(-) mice were exposed to hyperoxia for survival analysis or for sample collection after 72 h. This study was conducted under the guidelines recommended by the Osaka Medical College Ethical Association for Accreditation of Laboratory Animal Care.

Exposure of mice to hyperoxia

Mice were exposed to > 95% oxygen in a sealed chamber that was large enough to allow exposure of all the groups of mice simultaneously. The oxygen concentration during exposure was monitored continuously with an oxygen monitor (Jikco, Tokyo, Japan) and was maintained above 95% with input of 100% oxygen. Mice were allowed free access to food and water during exposure to hyperoxia and the chamber was opened for 5 min or less during removal of dead mice. In the survival experiment, exposure to hyperoxia was continued until all of the mice died. In the other experiment for sample collection, the duration of hyperoxia was 72 h. Hyperoxic exposure for sample collection was conducted in WT and KO E(-) mice.

Harvesting and preparation of samples

After 72 h of hyperoxia, WT and KO E(-) mice were sacrificed to harvest various samples. As a room air control, mice from each group that had only been exposed to room air were also sacrificed. The number of mice from which samples were harvested was

Table I. Composition of the diets.

Composition of diets (%)		Minerals (mg/100 g diet)		Vitamins (mg/100 g diet)	
Water	8.0	Calcium	892.63	Vitamin A (500,000 IU/g)	2.4
Crude protein	20.4	Phosphorus	664.57	Vitamin D_3 (100,000 IU/g)	2.4
Crude fat	6.0	Potassium	497.03	Vitamin K_3	0.3
Crude fiber	3.0	Magnesium	78.88	Vitamin B_1	1.5
Crude ash	6.2	Sodium	236.16	Vitamin B_2	1.56
Nitrogen-free extract	56.4	Iron	31.67	Vitamin B_6	1.02
		Zinc	3.46	Vitamin B_{12} (0.1%)	5.0
Total Energy, kcal/g	3.61	Copper	0.32	Biotin (2%)	0.5
		Cobalt	0.10	D-Calcium pantothenate	4.0
Ingredients (%)		Manganese	3.51	Para-aminobenzoic acid	10.15
				Nicotinic acid	10.15
Casein (vitamin-free)	24.500			i-Inositol	15.0
Cornstarch	45.500			Folic acid	0.2
Lard	6.0			Cholinium chloride	638.22
Sucrose	10.000				
Crystalline cellulose	3.000			D-alpha-tocopheryl acetate	
Powdered cellulose	2.000			Vitamin E-free diet	<0.100
Gelatinized starch	1.0			Control diet	10.000
Vitamin mixture	1.000				
Mineral mixture	7.000				

three-to-five in each group. Following induction of anaesthesia (pentobarbital at 50 mg/kg intraperitoneally), mid-line laparotomy was performed and the trachea was cannulated for continuous ventilation. Then blood was collected from the inferior vena cava into a heparinized syringe. Lavage of the lungs was done by repeated instillation and aspiration of saline ($0.5\ \text{ml} \times 5$). The bronchoalveolar lavage fluid (BALF) thus obtained was cooled on ice for cell counting and analysis of its protein concentration. Next, thoracotomy was done and the right ventricle was punctured with a 23 gauge butterfly needle, after which the pulmonary vessels were flushed with phosphate-buffered saline until the lungs became pale. Subsequently, the heart and large airways were dissected from the lungs and discarded. The lower lobe of the right lung was removed and immersed in 10% formalin to evaluate histologic changes. The other lung lobes were also removed and rapidly frozen in liquid nitrogen for storage at -80°C until analysis.

Lung histology

Formalin-fixed lung tissues were embedded in paraffin and three sections of each lung were cut and mounted onto slides. These sections were stained with hematoxylin and eosin and three fields per section (total nine fields per animal) were examined under a light microscope at $\times 400$ magnification. The severity of lung injury was quantified by a scoring system based on the method described by Arkovitz et al. [1]. Every field was scored on a scale from 0 (normal) to 5 (most severe) by a blinded reviewer who assessed each of the following three characteristics: interstitial cellularity, thickening of the alveolar septa (interstitial oedema) and atelectasis.

Analysis of BALF

Immediately after BALF collection, nucleated cells were stained with Samson's solution and the total nucleated cell count was determined by light microscopy. The remaining BALF was centrifuged for 10 min at 1000 rpm and the protein concentration of BALF supernatant was measured by the Bradford method with a commercial protein assay (Bio-Rad, Hercules, CA) according to the manufacture's instructions, using bovine serum albumin as the standard. Immediately after centrifugation, the remaining BALF supernatant was divided into microtubes under a stream of nitrogen gas to avoid further oxidation and was stored at -80°C until measurement of oxidative stress markers.

Lung tissue homogenate

Frozen lung tissue was homogenized in nine volumes of PBS containing 0.01% butylated hydroxytoluene (BHT). To avoid further oxidation of tissue samples, nitrogen gas was added to the buffer ~ 1 h before homogenization and the sample tube was kept under nitrogen during the process of homogenization. Lung tissue homogenates generated by this method were immediately used for measurement of oxidative stress markers and α-tocopherol. The remaining samples were centrifuged for 15 min at 10 000 rpm and the supernatants were used for measurement of proinflammatory cytokines and the protein concentration. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured by commercial kits (Endogen Mouse Interleukin-1β ELISA Kit, Endogen Mouse Interleukin-6 ELISA Kit; Endogen, Cambridge, MA). The protein concentration of the lung homogenates was measured by the method described above and

was used for correction of the other data from lung homogenate.

Analysis of α-tocopherol

The concentration of α-tocopherol in plasma and lung tissue homogenates was measured as described previously [35]. Samples were mixed with ethanol and pyrogallol to prevent further oxidation and all-*rac*-tocol was added as the internal standard. Then KOH was added and incubation was done for saponification. After cooling, hexane was added to the mixture and then the hexane layer was removed by evaporation under nitrogen. The residue was dissolved in ethanol and injected into the HPLC column, after which α-tocopherol was detected with an electrochemical detector (Shiseido, Tokyo, Japan). An LC-18 column (Supelco, Tokyo, Japan) was used and the eluent was methanol/$NaClO_4$. The α-tocopherol concentration was calculated by comparing the area under the curve (AUC) for a sample with that for the internal standard.

RNA extraction and real-time RT-PCR

Total RNA was extracted from frozen lung tissue by the guanidinium thiocyanate-phenol-chloroform method with Isogen reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions and was quantified by measuring the absorbance at 260 nm. Then reverse transcription to obtain cDNA was done with an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). Subsequently, RT reaction mixtures were amplified by LightCycler PCR using LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Switzerland) in accordance with the manufacturer's instructions. The oligonucleotide sequences of the cDNA primers were designed at Nihon Gene Research Laboratories (Nihon Gene Research Lab. Inc., Sendai, Japan). Primer sets and PCR product sizes for the mouse IL-1β, IL-6 and β-actin genes were as follows: IL-1β, forward, 5′-ATC AAC AAG AGC TTC AGG C-3′; IL-1β, reverse, 5′-GGT TAT CAT CAT CAT CCC A-3′(172 bp); IL-6, forward, 5′-GCT ATG AAG TTC CTC TCT GC-3′; IL-6, reverse, 5′-AGT GGT ATC CTC TGT GAA GTC T-3′(123 bp); β-actin, forward, 5′-CGT TGA CAT CCG TAA AGA CC-3′; β-actin, reverse, 5′-CCA CCG ATC CAC ACA GAG TA-3′(171 bp). The PCR products were verified by DNA sequencing. External PCR standards were employed to create standard curves and were generated by cloning the PCR product of cDNA from the liver of a wild-type mouse into the TOPO-TA vector (Qiagen, Valencia, CA). Using LightCycler software, the amplification curves of the experimental samples were plotted against these standard curves to obtain an estimate of the number of specific mRNA copies. To compensate for differences in RT efficiency among the samples, the values for IL-1β and IL-6 were then corrected by the copy number for β-actin.

Analysis of oxidative stress markers in lung tissue and BALF

Analysis of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and hydroxyoctadecadienoic acid (HODE) in lung tissue and BALF was performed by the method reported previously [36]. Immediately after homogenization or thawing, the internal standard for 8-iso-$PGF_{2\alpha}$-d4 (Wako, Osaka, Japan) and methanol were added to an aliquot of homogenized lung or BALF, followed by the reduction of hydroperoxides with sodium borohydride. Then the reduced samples were saponified with KOH. After acidification with HCl, solid-phase extraction was performed with C18 and NH2 cartridges. The extracted samples were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and were injected into a gas chromatograph (GC 6890 N, Agilent Technologies Co. Ltd.) equipped with a quadrupole mass spectrometer (5973 Network, Agilent Technologies Co. Ltd.). Identification of HODE and 8-iso-$PGF_{2\alpha}$ was done on the basis of their retention times and mass patterns. Specific ions were selected for quantification of HODE and 8-iso-$PGF_{2\alpha}$, using the internal standard. Accordingly, 9-(E, Z)- and 13-(Z, E)-HODE, 9-(E, E)-HODE, 13-(E, E)-HODE and 8-iso- $PGF_{2\alpha}$ were measured and isolated by GC-MS.

Analysis of fatty acids in lung

For correction of the HODE and 8-iso-$PGF_{2\alpha}$ content of lung tissue, we evaluated the pre-oxidized levels of fatty acids, arachidonate(20:4) and linoleate(18:2), respectively. These fatty acids were analysed by the method described previously [37]. Chloroform/methanol extraction of lung tissue samples was followed by evaporation and the residue was dissolved in methanol/benzene containing the internal standard. Then this solution was methanolysed with acetyl chloride. After neutralization, the benzene layer was injected into the gas chromatograph and detection of fatty acids was done with a flamed ionized detector. The amount of each fatty acid was quantified by comparing its peak area with that of the internal standard.

Statistical analysis

Data are expressed as the mean ± SE. Statistical analysis were done by using Statview software (StatView version 5.0, SAS Institute, NC). Differences between two groups were evaluated by the Mann-Whitney U-test and survival curves were compared by log-rank test. Differences between mean values were considered significant at $p < 0.05$.

Results

Baseline plasma and lung tissue levels of α-tocopherol

First, we investigated the baseline plasma and lung tissue levels of α-tocopherol in each group. Plasma and lung α-T levels decreased gradually in the order of WT > WT E(-) > KO > KO E(-) mice, and the differences between each group were significant (Table II).

Survival during hyperoxia

The effect of vitamin E deficiency on survival during hyperoxia was investigated by Kaplan-Meier analysis (Figure 1). During hyperoxia, KO E(-) mice ($n = 8$) began to die after 40 h and all of the animals were dead by 96 h, whereas WT mice ($n = 22$) began to die after 64 h and some animals remained alive at 104 h. The survival rate of KO E(-) mice was significantly lower compared with that of WT mice after 72 h of hyperoxia ($p = 0.0018$, by the log-rank test). The difference of survival between the two groups was maximal at this time, so we selected it for our subsequent time-course experiment.

Changes of plasma and lung tissue levels of α-tocopherol due to hyperoxia

After exposure to hyperoxia, plasma α-T levels were almost unchanged, whereas lung α-T levels were decreased significantly in both WT and KO E(-) mice. The lung α-T level was reduced by 26% in WT mice and by 47% in KO E(-) mice (Table III).

Lung histology

Before hyperoxia, the lungs were almost normal in KO E(-) mice as well as WT mice (Figure 2A and B). After 72 h of hyperoxia, interstitial cellular infiltration and oedema (thickening of alveolar septa) were seen in the lungs from both groups of mice, but the changes were more severe in KO E(-) mice than WT mice. In addition, WT mice still had almost normal alveolar architecture, whereas KO E(-) mice showed loss of air spaces due to collapse of alveoli or severe alveolar wall thickening (Figure 2C and D).

Table II. Baseline plasma and lung α-T levels in each group.

	Plasma α-T (nmol/ml)	Lung α-T (nmol/g protein)
WT	11 ± 0.5	408 ± 37
WT E(-)	1.3 ± 0.2⋆	217 ± 85⋆
KO	0.5 ± 0.02⋆†	28 ± 2.8⋆†
KO E(-)	0.4 ± 0.05⋆†§	9.9 ± 0.4⋆†§

Values are the mean ± SE; WT = wild -type mice fed a normal diet (n = 5); WT E(-) = wild-type mice fed a vitamin E-deficient diet (n = 5); KO = a-TTP knockout mice fed a normal diet (n = 3); KO E(-) = a-TTP knockout mice fed a vitamin E-deficient diet (n = 4); ⋆ p < 0.05 vs. WT. †p < 0.05 vs. WT E(-). § p < 0.05 vs. KO.

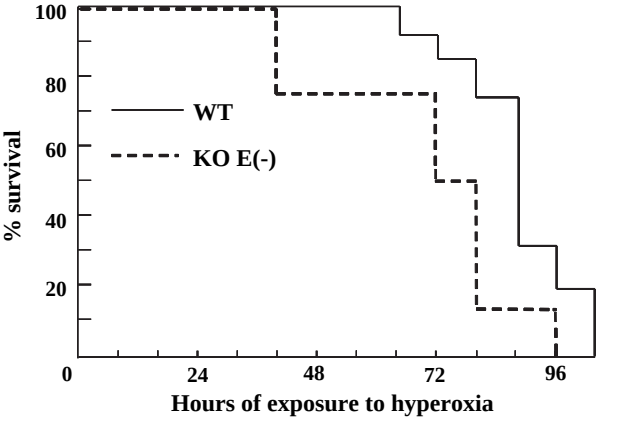

Figure 1. Survival rate of KO E(-) vs WT mice during hyperoxia. Survival of KO E(-) mice was significantly lower than that of WT mice after 72 h of hyperoxia. ⋆ $p = 0.0018$ by the log-rank test. WT = wild-type mice fed a normal diet ($n = 22$); KO E(-) = α-TTP knockout mice fed a vitamin E deficient diet ($n = 8$).

Before hyperoxia, the lung injury score was not significantly different between the two groups of mice and the median score for every characteristic was less than 1 in both groups (data not shown). After hyperoxia, the score increased significantly in both groups, but the median score of KO E(-) mice was significantly higher than that of WT mice for every characteristic as well as the total score (Table IV).

Total cell count and protein content of BALF

To assess inflammatory cells infiltration into the alveoli and changes of pulmonary capillary permeability due to hyperoxia, we measured the total cell count and protein concentration of BALF before and after 72 h of hyperoxia. The total cell count of KO E(-) mice was only slightly higher than that of WT mice at baseline. After 72 h of hyperoxia, the total cell count increased significantly in both groups, but the BALF cell count of KO E(-) mice was significantly higher than that of WT mice (Figure 3A; 40 ± 1 vs 65 ± 15, $p = 0.049$). Like the cell count, the protein concentration of BALF was increased significantly after hyperoxia in both groups. Although protein levels were almost the same at baseline (Figure 3B),

Table III. Plasma and lung a-T levels of WT and KO E(-) mice before and after 72 hr of hyperoxia.

		Plasma α-T (nmol /ml)	Lung α-T (nmol/g protein)
WT	0 hr	11 ± 0.5	408 ± 37 †
	72 hr	9.7 ± 0.6	301 ± 25
KO E(-)	0 hr	0.4 ± 0.1⋆	9.9 ± 0.4⋆ †
	72 hr	0.4 ± 0.2⋆	5.2 ± 0.7⋆

Values are the mean ± SE; WT = wild-type mice fed a normal diet; KO E(-) = a-TTP knockout mice fed a vitamin E-deficient diet; Samples were harvested from 3–5 mice in each group.
⋆p < 0.05 vs. WT at each time point. †p < 0.05 vs. 72 hr in each group.

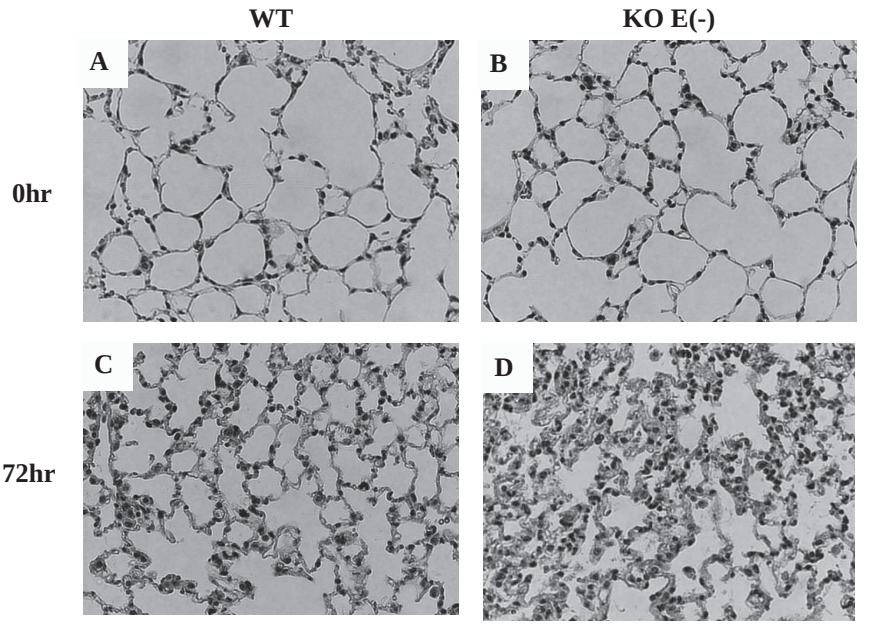

Figure 2. Lung histology in WT and KO E(-) mice before and after 72 h of hyperoxia. Before hyperoxia, lung morphology is almost normal in WT mice (A) and KO E(-) mice (B). After 72 h of hyperoxia, interstitial cellular infiltration and oedema are seen in the lungs of mice from both groups, but the changes are more severe in KO E(-) mice (D) than WT mice (C). KO E(-) mice show loss of air spaces due to collapse of alveoli or severe thickening of the alveolar walls. WT = wild-type mice fed a normal diet; KO E(-) = α-TTP knockout mice fed a vitamin E-deficient diet. Magnification: $\times 400$. Scale bar: 100 μm. Photomicrographs are representative findings ($n = 3$ per group).

the level after 72 h of hyperoxia was significantly higher in KO E(-) mice than in WT mice (69 ± 4 vs 131 ± 4, $p = 0.025$).

Expression of proinflammatory cytokines in lung tissue

The inflammatory response to hyperoxia was evaluated by measuring protein and mRNA expression for two proinflammatory cytokines by ELISA and RT-PCR, respectively. The levels of IL-1β and IL-6 protein did not change significantly after hyperoxia and differences between the two groups were also not significant (data not shown). IL-1β mRNA expression decreased significantly in both groups after 72 h of hyperoxia (Figure 4A). IL-1β mRNA expression was higher in KO E(-) mice than in WT mice from baseline until 72 h of hyperoxia, but the difference between the two groups was only significant after hyperoxia (0.0069 ± 0.0021 vs 0.0014 ± 0.0003, $p = 0.014$). IL-6 mRNA expression in the lungs of KO E(-) mice showed an increase during 72 h of hyperoxia, whereas it was almost unchanged in WT mice (Figure 4B). After 72 h of hyperoxia, IL-6 mRNA expression was significantly higher in KO E(-) mice than in WT mice (0.5 ± 0.2 vs 1.7 ± 0.7, $p = 0.050$).

Table IV. Lung injury scores of WT and KO E(-) mice after 72 hr of hyperoxia.

Characteristic	WT	KO E(-)	P value
Interstitial cellularity	2.2 ± 0.1	3.0 ± 0.2	0.040
Interstitial edema	2.7 ± 0.2	3.6 ± 0.2	0.001
Atelectasis	1.8 ± 0.1	2.4 ± 0.1	<0.0001
Total	6.7 ± 0.3	9.0 ± 0.4	0.0002

Values are the mean $\pm$ SE ; n = 3 mice per group. n = 27 fields for each mouse. For each characterisic , every field was scored on a scale from 0 (normal) to 5 (most severe).

Oxidative stress markers in lung tissue and BALF

We employed two oxidative stress markers to evaluate the generation of ROS in lung tissue during hyperoxia. 8-Iso-$PGF_{2\alpha}$ is a prostaglandin F_2-like compound that is formed specifically by free radical-mediated oxidation of arachidonate and is regarded as the gold standard for assessment of oxidative injury *in vivo* [38]. The other oxidative stress marker chosen for this study was HODE, which is a chemically stable final metabolite of oxidized linoleate (hydroperoxyoctadecadienoate:HPODE). HODE has four stereoisomers, among which 9- and 13-(E, E)-HODE are specific markers of free radical-mediated lipid peroxidation [39], and it has recently been used as a marker of *in vivo* oxidative stress [40,41]. Both 8-iso-$PGF_{2\alpha}$ and HODE levels in lung tissue were corrected for their parent molecules (arachidonate (20:4) and linoleate (18:2), respectively). We found that 8-iso-$PGF_{2\alpha}$ levels in lung tissue and BALF were increased significantly in both groups after 72 h of hyperoxia (Figure 5A and C). At 72 h, KO E(-) mice had significantly higher levels in both lung tissue and BALF than WT mice (136 ± 49 vs 42 ± 10, $p = 0.014$ in lung tissue; 9.4 ± 2.0 vs 26 ± 8, $p = 0.016$ in BALF, respectively), while the baseline values for both were similar in each group. These findings indicate that

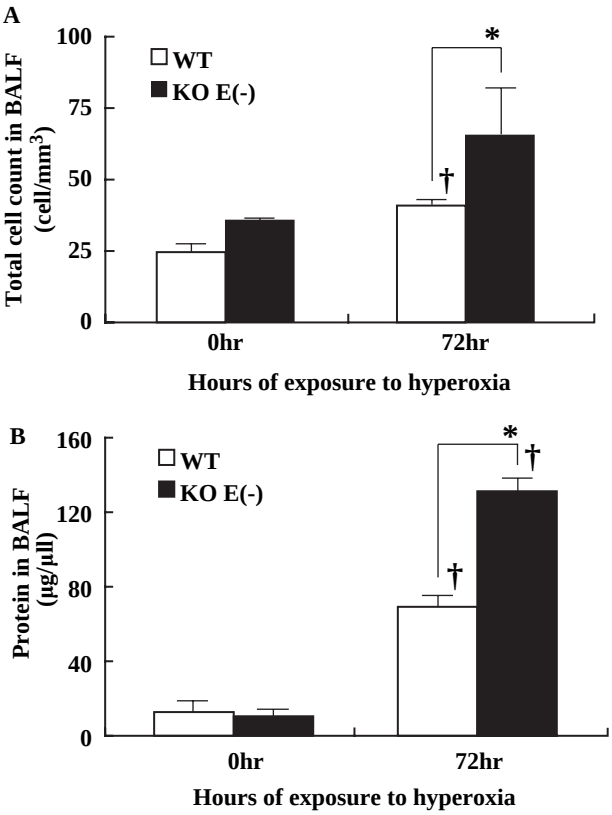

Figure 3. Changes of the total cell count (A) and protein concentration (B) in BALF after mice were exposed to hyperoxia for 72 h. Both the total cell count and the protein concentration of BALF from each group were increased significantly by hyperoxia and the values of KO E(-) mice were significantly higher than those of WT mice. Data are expressed as the mean ± SE. Open bars = wild-type mice fed a normal diet (WT). Closed bars = α-TTP knockout mice fed a vitamin E-deficient diet (KO E(-)). *$p < 0.05$, vs 72 h WT. †$p < 0.05$, vs 0 h in each group.

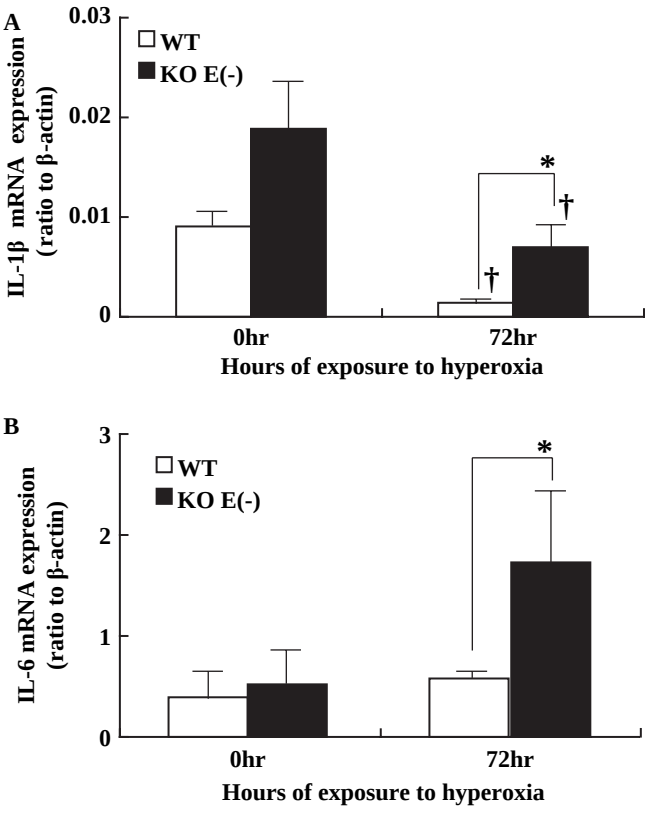

Figure 4. Changes of proinflammatory cytokine mRNA expression in the lung tissue of mice exposed to hyperoxia for 72 h. Relative IL-1β mRNA expression in the lungs was decreased significantly in both groups after 72 h of hyperoxia (A). IL-1β mRNA expression in KO E(-) mice was higher than in WT mice both before and after hyperoxia. Relative IL-6 mRNA expression in the lungs of KO E(-) mice showed an increase during 72 h of hyperoxia, whereas that of WT mice was almost unchanged (B). At 72 h, IL-6 mRNA expression in KO E(-) mice was significantly higher than in WT mice. Data are shown as the mean ± SE. Open bars = wild-type mice fed a normal diet (WT). Closed bars = α-TTP knockout mice fed a vitamin E-deficient diet (KO E(-)). *$p < 0.05$, vs 72 h WT. †$p < 0.05$, vs 0 h in each group.

vitamin E deficiency exacerbates oxidative stress in the lungs (or at the alveolar surface) due to hyperoxia. On the other hand, the 9, 13-(E, E)-HODE level of lung tissue showed no increase in either group due to hyperoxia (Figure 5B). The BALF level of 9, 13-(E, E)-HODE increased after hyperoxia in both groups, but a significant change was only observed among WT mice (Figure 5D). At 72 h, the values of the two groups were not significantly different.

Discussion

When we examined the survival of α-TTP knockout mice fed a normal diet (KO mice) compared with that of wild-type mice during exposure to hyperoxia, the survival rates of the two groups were not significantly different. Therefore, we gave α-TTP knockout mice a vitamin E-free diet for 1 month (KO E(-) mice) and then compared their survival with that of the other mice (wild-type mice fed a normal diet (WT), WT E(-) and KO mice) after exposure to hyperoxia. KO E(-) mice showed significantly lower vitamin E levels than the other mice in both plasma and lung tissue. In addition, KO E(-) mice exhibited a significantly lower survival rate than WT mice (the differences of survival rates were not significant between the other pairs of all four groups, data not shown). Based on these results, we thought that the lower survival of KO E(-) mice during hyperoxia might have been due to more severe pulmonary vitamin E deficiency compared with that in KO mice. Therefore, we used KO E(-) mice as a severe vitamin E deficiency model for comparison with WT mice in our subsequent time-course experiment on hyperoxia. This experiment revealed that severe vitamin E deficiency exacerbates acute hyperoxic lung injury, inflammation and oxidative stress.

In the present study, it was noteworthy that the baseline lung α-T levels of KO E(-) mice was more than 60% lower than that in KO mice, although there was little difference of baseline plasma α-T levels. α-T kinetics in the body, especially the lungs, are not fully

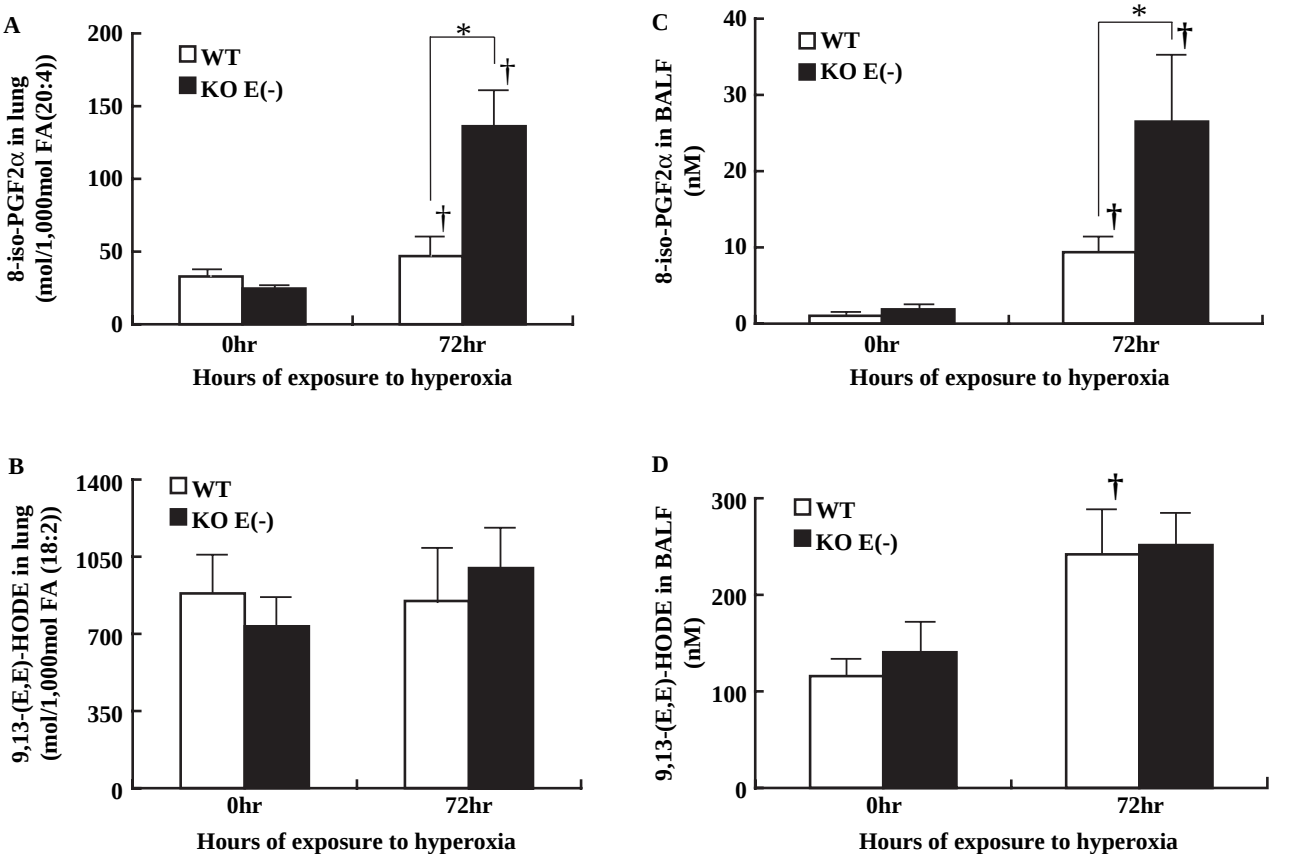

Figure 5. Changes of oxidative stress markers in lung tissue or BALF from mice exposed to hyperoxia for 72 h. The 8-iso-$PGF_{2\alpha}$ level in lung tissue and BALF was increased significantly in both groups after 72 h of hyperoxia (A and C). At 72 h, KO E(-) mice exhibited significantly higher levels in both lung tissue and BALF than WT mice. In contrast, the lung level of 9, 13-(E, E)-HODE showed no increase in both groups due to hyperoxia (B). The BALF level of 9, 13-(E, E)-HODE increased after hyperoxia in both groups, but a significant change was only observed in WT mice (D). At 72 h, the values of the two groups were not significantly different. Data are shown as the mean ± SE. Open bars = wild-type mice fed a normal diet (WT). Closed bars = α-TTP knockout mice fed a vitamin E-deficient diet (KO E(-)). *$p < 0.05$, vs 72 h WT. † $p < 0.05$, vs 0 h in each group.

understood. However, the uptake, secretion and intracellular transport of α-T by alveolar cells are thought to be associated with some specific proteins. Pulmonary uptake of α-T is mainly mediated by high density lipoprotein (HDL) as the source of vitamin E in the blood and scavenger receptor class B type 1 (SR-B1) as the specific HDL receptor expressed by alveolar type II cells [42]. ATP binding cassette 1 (ABCA1) has an important role in surfactant secretion and may also be important for α-T secretion at the alveolar surface [43]. In addition, α-TTP is known to be expressed in the lungs [44]. The pulmonary role of α-TTP is not understood, but it might be associated with local α-T kinetics. Recently, Valacchi et al. [44] reported that protein or gene expression of SR-B1, ABCA1 and α-TTP in the lungs was differentially affected by age and environmental oxidants. In addition, α-TTP gene expression in the liver is affected by the α-T nutritional status [45] and this might also be true for the lungs. The lower baseline lung α-T level in KO E(-) mice might be induced by differences of the baseline α-T level and/or α-TTP gene deletion itself and/or interactions of the proteins mentioned above in response to alimentary vitamin E depletion.

After hyperoxic exposure, plasma α-T levels were almost unchanged in both the WT and KO E(-) mice, whereas lung α-T levels showed a significant decrease in both groups. These findings might be explained by abundant ROS production in the lungs during hyperoxia. In addition, the absolute reduction of lung α-T due to hyperoxia was smaller in KO E(-) mice than WT mice, whereas the increase of 8-iso-PGF2α in the lungs was greater. In KO E(-) mice, ROS production associated with hyperoxia might overwhelm the scavenging ability of residual α-T, whereas WT mice had sufficient α-T to cope with hyperoxia.

Our main findings are consistent with those of a recent study by Shvedova et al. [46] using vitamin E-deficient mice exposed to an inhalant with both oxidative and proinflammatory properties. Their acute lung injury model was induced by single-walled carbon nanotubes (SWCNTs), which are known to

induce both pulmonary inflammation and oxidative stress. After aspiration of SWCNTs, the lungs of mice fed a vitamin E-deficient diet showed a significant increase of lipid peroxidation and inflammation with a decrease of antioxidants compared to the lungs of control mice. Thus, vitamin E deficiency seems to exacerbate pulmonary oxidative stress and inflammation as well as lung injury, leading to death after exposure to inhalants that induce oxidative stress and inflammation. Contrary to our findings, however, previous studies showed at best a partial effect of α-T administration on hyperoxic lung injury models [30–32]. One possible reason is that the dose of vitamin E necessary to protect alveolar membranes or surfactant lipids from hyperoxia might not need to be greater than the physiological concentration. A classical study by Tappel [47] showed that the molar ratio of vitamin E to polyunsaturated fatty acids is 1:1000 for adequate antioxidant protection of membranes. If vitamin E levels fall below this molar ratio, antioxidant activity is diminished. Another possibility is that vitamin E deficiency might interfere with pulmonary function in ways unrelated to its antioxidant properties. Recent studies have shown that vitamin E deficiency diminishes surfactant lipid biosynthesis by type II alveolar pneumocytes [48]. Moreover, hyperoxia is known to diminish surfactant secretion into the alveoli [49]. Therefore, vitamin E deficiency might accelerate respiratory failure due to alveolar congestion during hyperoxia independently of any diminished antioxidant or anti-inflammatory activity.

In the present study, IL-6 mRNA expression in the lungs of KO E(-) mice increased significantly more than WT mice after hyperoxia. In contrast, IL-1β mRNA expression decreased significantly in the lungs of both groups after hyperoxia, although the values in KO E(-) mice were always higher than in WT mice from baseline onwards (significant differences were only seen after hyperoxia). In general, IL-1β is regarded as an 'early response cytokine' and is known to be secreted initially in response to various insults [42]. Thus, IL-1β mRNA levels in lung tissue might have reached an earlier peak and then decreased by 72 h, while remaining higher in KO E(-) mice than in WT mice.

We also found that lung tissue and BALF levels of 8-iso-$PGF_{2\alpha}$ were increased significantly in KO E(-) mice compared with WT mice after hyperoxia. On the other hand, the 9, 13-(E, E)-HODE in levels of both lung tissue and BALF did not exhibit any significant differences between the two groups of mice before or after hyperoxia. One possible explanation for this discrepancy is that the response to hyperoxic oxidative stress might be different between these two markers. F2-isoprostane is a free radical-mediated oxidative product of arachidonate and is regarded as the gold standard *in vivo* oxidative stress marker. Indeed, arachidonate is $\sim$3-times more reactive than linoleate with peroxyl radicals [50]. Secondly, the metabolic rate might differ between these two markers. Intracellular ROS production during hyperoxia occurs over several hours [51] and ROS might oxidize tissue lipids after a shorter time than 72 h. Therefore, HODE might have increased in lung tissue before 72 h of hyperoxia and then may have been metabolized more rapidly than 8-iso-$PGF_{2\alpha}$. Thirdly, differences in the composition or distribution of fatty acids among organs might influence local production of lipid peroxides during hyperoxia. Generally, linoleate is the most abundant polyunsaturated fatty acid *in vivo*, but Dilley et al. [33] reported that rat lungs contained almost the same amount of arachidonate and linoleate. They also showed that the fatty acid composition of lung organelles was influenced by both hyperoxia and vitamin E deficiency. In the present study, the baseline values of these fatty acids in lung tissue were not significantly different between WT and KO E(-) mice. After hyperoxia, both fatty acids increased in WT mice and decreased in KO E(-) mice (data not shown).

The present study has the limitation that investigation of the influence of vitamin E deficiency on hyperoxic lung injury may have been influenced by the different strains of mice, which could have a potential to exhibit different responses to inflammatory and oxidative stresses. Accordingly, hyperoxic challenges should be carried out using the same strain of mice in the future.

In summary, the present study provides new information about the deleterious effect of vitamin E deficiency on acute hyperoxic lung injury. Oxidative stress and pulmonary inflammation due to hyperoxia were also enhanced by vitamin E deficiency. This study did not focus on the mechanism by which vitamin E deficiency exacerbates acute hyperoxic lung injury. To elucidate the factor initially influenced by vitamin E deficiency during hyperoxia, changes at earlier time points would need to be studied. Further studies using cultured cells might be needed to obtain more detailed information about the changes of intracellular signalling during hyperoxia related to vitamin E deficiency.

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