Animal Cells and Systems 13: 105-112, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Changes in Cytochrome c Oxidase and NO in Rat Lung Mitochondria Following Iron Overload

Misun Kim, Min-a Hong, and Eunsook Song*

Division of Life Science, College of Natural Sciences, Sookmyung Women's University, Seoul 140-742, Korea

Abstract: In this study, the effects of iron on cytochrome c oxidase (CcO) in rat lung mitochondria were examined. Similar to liver mitochondria, iron accumulated considerably in lung mitochondria (more than 2-fold). Likewise, the reactive oxygen species and nitric oxide (NO) content of mitochondria were increased by more than 50% and 100%, respectively. NO might be produced by nitric oxide synthase (NOS), eNOS and iNOS type, with particular contribution by NOS in mitochondria. The respiratory control ratio of iron-overloaded lung mitochondria dropped to nearly 50% due to increased state 4. Likewise, cytochrome c oxidase activity was lowered significantly to approximately 50% due to excess iron. Real-time PCR revealed that the expression of isoforms 1 and 2 of subunit IV of CcO was enhanced greatly under excess iron conditions. Taken together, these results show that oxidative phosphorylation within lung mitochondria may be influenced by iron overload through changes in cytochrome c oxidase and NO.

Key words: lung mitochondria, iron overload, cytochrome c oxidase subunit IV, nitric oxide, nitric oxide synthase

INTRODUCTION

Iron is very efficient at oxidoreduction and essential for all living organisms. Reactive iron that is chelatable, called 'labile iron' (Kakhlon and Cabantchik, 2002), is maintained at low levels inside cells. However, an excessive amount of iron can elicit overproduction of oxygen radical in aerobic species (Gutteridge et al., 2001). Several disease conditions that result in pulmonary dysfunction have been linked to iron overload, including thalassemia major, lung fibrosis, and interstitial edema (Factor et al., 1994; Carnelli et al., 2003). Moreover, iron is considered a leading cause of lung injury following asbestos exposure (Kamp et al., 2002).

The lung is unique in maintaining its function under the atmospheric pressure of oxygen. Even normoxia is detrimental to lung tissue (Claireaux, 1975), which should be modulated by nitric oxide (NO) (Barnes and Belvisi, 1993). NO, which is released by macrophages, induces iron secretion from target cells, resulting in a cytostatic effect (Nestel et al., 2000). Conversely, intraperitoneal administration of iron increases the blood NO level in the presence of lipopolysaccharide (LPS) in rats (Galleano et al., 2004). These reports reveal that iron levels are regulated by NO, which can bind free labile iron to form a nitrosyl iron complex that is secreted from the cell (Lancaster and Hibbs, 1990). This interrelated metabolism of iron and NO suggests that the latter may be involved in causing human diseases associated with iron-overload.

Recent studies indicate that iron is targeted to the mitochondria, indicating that this organelle plays a key role in iron-related pathophysiology (Napier et al., 2005). Interestingly, published reports have demonstrated that NO modulates mitochondrial respiration by specific inhibition of cytochrome c oxidase (also known as Complex IV within the electron transport system) (Brown and Cooper, 1994; Cleeter et al., 1994). These independent findings highlight mitochondria as the primary functional location within the cell.

Despite increasing clinical evidence for iron-related pulmonary dysfunction, few studies have been performed to explore the underlying mechanisms that cause these conditions. Based on published results, we postulate that mitochondria, as target organelles of iron, may be altered due to iron overload. Changes in CcO and NO resulting from excessive iron have been examined as iron-induced NO binds CcO directly. In this study, we measured reactive

Abbreviations: RCR, respiratory control ratio; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; NOS, nitric oxide synthase; CcO, cytochrome c oxidase; RT-PCR, real-time polymerase chain reaction

*To whom correspondence should be addressed.
Tel: +82-2-710-9417; Fax: +82-2-2077-7322
E-mail: eunsong@sookmyung.ac.kr

oxygen species (ROS), NO content, and CcO activity to assess the effect of iron overload on mitochondria.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from ORIENT Biology Inc. (Korea). All groups were raised in the same facility, received the same diet *ad libitum*, and were studied simultaneously. For the iron overload treatments, male rats (~260-280 g) were injected with $FeCl_3$ (0.049 mg/g) hypodermically every other day for two weeks for a total of six injections. As a control, rats were injected with 0.15 M saline instead of $FeCl_3$ under the same conditions.

Isolation of lung mitochondria

Excised tissues were washed with 0.25 M cold sucrose solution and minced into small pieces in isolation buffer (20 mM Tris-Cl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.5% protease inhibitor cocktail (Sigma, St. Louis, MO, USA), 1 mM NaF, 1 mM Na_3VO_4). Minced tissues were homogenized with a Teflon homogenizer as described by Pande and Blanchaer (1971). The homogenate was centrifuged, and the pellet was resuspended in isolation buffer. After washing twice, the pellet was used immediately or kept at −80°C until use.

Measurement of iron content

Iron content was measured according to Levi *et al.* (1988). Mitochochondrial homogenate (500 μg) was added to 75 mM Na_2SO_3, 0.05%(v/v) bipyridyl, and 6%(v/v) acetic acid. After boiling for 90 min, the mixtures were cooled and centrifuged to discard the denatured proteins. The absorbance of the supernatant was measured at 520 nm by a UV 2550 double beam spectrophotometer (Shimazu, Japan).

Measurement of the respiratory control ratio

The respiratory control ratio (RCR) of isolated mitochondria was determined according to Estabrook (1967). Isolated mitochondria (1 mg) were suspended in a solution (1 mL) containing 0.25 M sucrose, 20 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 20 mM KCl, and 1 mM EDTA. The suspension was incubated in the oxygen electrode chamber (Hansatech, UK) at 25°C with circulating water. After 30 sec, 5 mM succinate was added as a respiratory substrate (state 2) followed by 1 mM ADP (state 3). The consumption of oxygen was monitored and the oxygen curve would stabilize after a few minutes (state 4). The RCR was calculated as the oxygen consumption at state 3 (in the presence of ADP) to that at state 4 (after ADP is converted to ATP).

Measurement of reactive oxygen species (ROS) and nitric oxide (NO)

ROS content was measured as described by Valkonen and Kuusi (1997) with some modification. Isolated mitochondria were suspended to 0.25 mg/mL in phosphate buffered saline (pH 7.4) prior to the addition of 330 μM 2'7'-dichlorodihydrofluorescein (DCF) diacetate probe immediately before measurement. The intensity was estimated at 504 nm. The production of NO was observed according to Kolb *et al.* (1994). Mitochondria (0.25 mg/mL) were mixed with Griess reagent containing 0.1% N-(1-naphthyl) ethylenediamine and 1% sulfanilamide for 10 min at 4°C. This mixture was measured at 550 nm.

Measurement of cytochrome c oxidase (CcO) activity

CcO activity was measured according to Arnold and Kadenbach (1997). The concentration of mitochondria (heme aa_3) for this assay was determined by using the following equation: (605 nm-630 nm)/24. Mitochondria (to 0.1 μM of heme aa_3) were incubated in 50 mM K-Pi (pH 7.4) and 1% Tween 20 in an oxygen electrode (Hansatech, UK) chamber at 25°C. After the incubation, 18.5 mM ascorbate (pH 8.0), 0.7 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), and 1, 2, 4, 6, or 10 μM cytochrome c was added. CcO activity was calculated by turnover number as described in the following equation:

$$TN = \frac{\text{consumed oxygen } (1/4\ O_2)\ (\mu M)}{\text{concentration of heme } aa_3\ (\mu M) * \text{time (sec)}}$$

RNA extraction

Total RNA was extracted from tissue according to Chomczynski and Sacchi (1987). Briefly, homogenate (50 mg) with liquid nitrogen was mixed with 1 mL TRIZOL (Invitrogen, Carlsbad, CA, USA) prior to the addition of 0.2 mL chloroform. After vigorous shaking by hand, the samples were incubated on ice for 15 min. Following centrifugation at 12,000×*g* at 4°C for 20 min, the colorless upper phase was obtained and mixed with an equal volume of isopropyl alcohol. After centrifugation for 10 min, the RNA pellet was washed twice with 75% ethanol. The pellet was dried briefly and then dissolved in RNase-free water. RNA with an A_{260}/A_{280} ratio ≥1.8 was used in the present study.

Real-Time PCR (RT-PCR)

To quantify COX IV-1 (isoform 1) and COX IV-2 (isoform 2) gene expression, real- time PCR (RT-PCR) was performed. Expression of citrate synthase, a housekeeping gene, was used as a control. cDNA were prepared using the First

Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Total RNA (500 ng) was added to a reaction solution containing 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 0.0225 OD units of oligo dT, 3.5 mg bovine serum albumin, 3 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates, 30 U RNase inhibitor, 500 U MMLV reverse transcriptase, and RNase-free water to 20 μL. Reactions were carried out by incubation at 70°C for 5 min, then at 37°C for 5 min, and 42°C for 60 min. A final incubation at 70°C for 10 min was performed to inactivate the reverse transcriptase. The primers used for the PCR are listed in Table 1. All PCR primers were synthesized by Cosmo (Seoul, Korea). RT-PCR was performed using a Rotor-Gene 3000 thermocycler (Corbett Robotics, Australia) with SYBR green (Qiagen, Chatsworth, CA, USA) as recommended by the manufacturer. Reactions were performed as follows: 95°C for 15 min, 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 60 sec at 72°C, then 10 min at 72°C. The mRNA levels were normalized to the corresponding citrate synthase level for each sample to adjust for uncontrolled variability between samples. Using the manufacturer's Rotor-Gene 6 software, the values were extrapolated to calculate the relative number of mRNA copies compared to citrate synthase.

Western blot analysis

Protein samples from lung homogenates or mitochondrial fractions were separated on 8% polyacrylamide gels and transferred onto PVDF (Pall Corporation, East Hills, NY, USA). The membranes were probed using anti-iNOS, anti-eNOS, anti-nNOS (BD Biosciences, San Jose, CA, USA), and anti-CcO IV (MitoScience, Eugene, OR, USA) antibodies at a 1:1,000 dilution followed by peroxidase-conjugated sheep anti-mouse IgG secondary antibody at 1:5,000 (Amersham Bioscience, Pittsburgh, PA, USA). As a control for protein loading, membranes were probed for Adenine Nucleotide Translocator (ANT) protein using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution. Chemiluminescence (Pierce, Rockford, IL, USA) was used for detection and densitometry using Multigause 3.0 (Fujifilm, Japan) was performed for quantification.

Table 1. Sequences of PCR primers for COX IV transcripts

Gene	Sequence of primer
COX IV-1	Forward primer 5'-CCTGAAGGAGAAGGAGAAGG-3'
	Reverse primer 5'-GCGAAGCTCTCGTTAAACTG-3'
COX IV-2	Forward primer 5'-GAGACCTTCGCAGAGATGAA-3'
	Reverse primer 5'-CCGTGAATCCAATGAAGAAG-3'
Citrate synthase	Forward primer 5'-GGTTGTCCCAGGATATGGTC-3'
	Reverse primer 5'-GGCAGATGTTTCAGAGCAAA-3'

RESULTS

Increases in iron content, ROS, and NO in mitochondria

For mitochondrial biogenesis to occur, iron is required for the respiratory enzymes involved in oxidative phosphorylation, including heme and the iron-sulfur center. Mitochondria are the primary sites of iron metabolism. Compared to the liver, the lung has fewer mitochondria. It is possible that this reduced quantity reflects the organ's adaption to a high oxygen environment and attempt at avoiding damage due to oxygen overexposure. Thus, iron content would be expected to be lower in the lung than in the liver (approximately 50% of that in liver) (Fig. 1). However, iron content increased more than 2-fold in an iron-overloaded lung and liver.

Since iron is an active generator of ROS, we proceeded to determine the level of oxygen free radicals. As expected, the level of ROS was augmented in the iron-overloaded lung and liver by greater than 50% (Fig. 2). Iron is known to induce the production of NO, which plays a role in facilitating iron transport by the nitrosylated iron complex (Lancaster and Hibbs, 1990). As can be seen in Fig. 3, NO in mitochondria also increased in parallel with iron excess in the lung and liver. The abundance of NO seems to interfere with oxygen or oxygen-related functions, including CcO activity.

Determination of NOS types in the lung

Three types of nitric oxide synthase (NOS) exist in mammalian tissues. To determine which is present in the

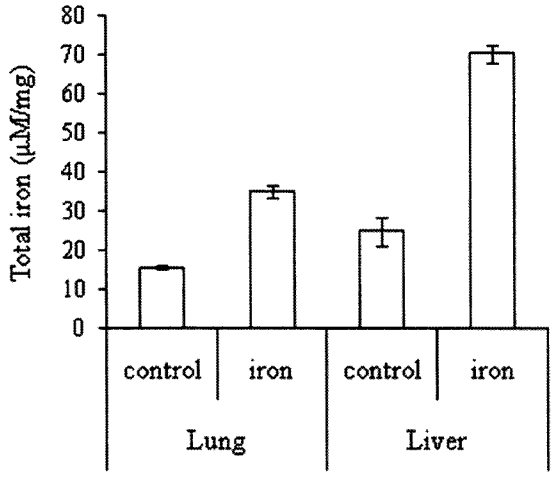

Fig. 1. The mitochondrial iron content in rat lung and liver. For the experimental group, iron (0.049 mg/g body wt) was injected six times every other day for 2 weeks. Total iron was measured from lung and liver mitochondria.

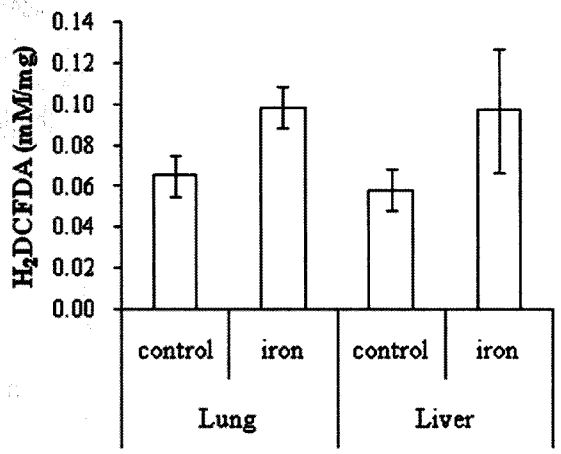

Fig. 2. Elevation of ROS in lung and liver mitochondria in iron-overloaded rat. To the isolated mitochondria in phosphate buffered saline (pH 7.4) (0.25 mg/mL), 0.1 mL of 330 μM 2'7'-dichloro-dihydrofluorescein (DCF) diacetate was added immediately before measurement, and the absorbance was recorded at 504 nm.

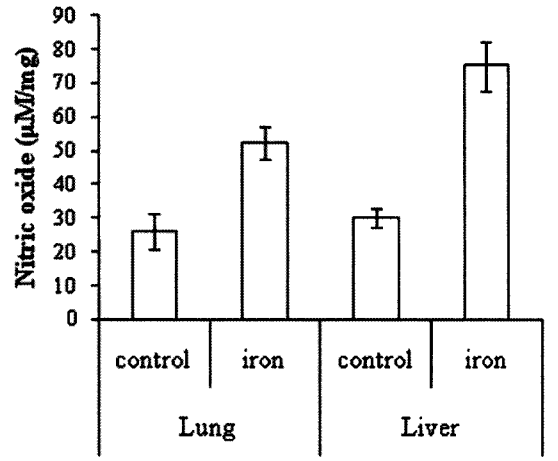

Fig. 3. Increased nitric oxide (NO) in the iron-overloaded lung and liver mitochondria. The production of NO was observed according to Kolb *et al.* (1994).

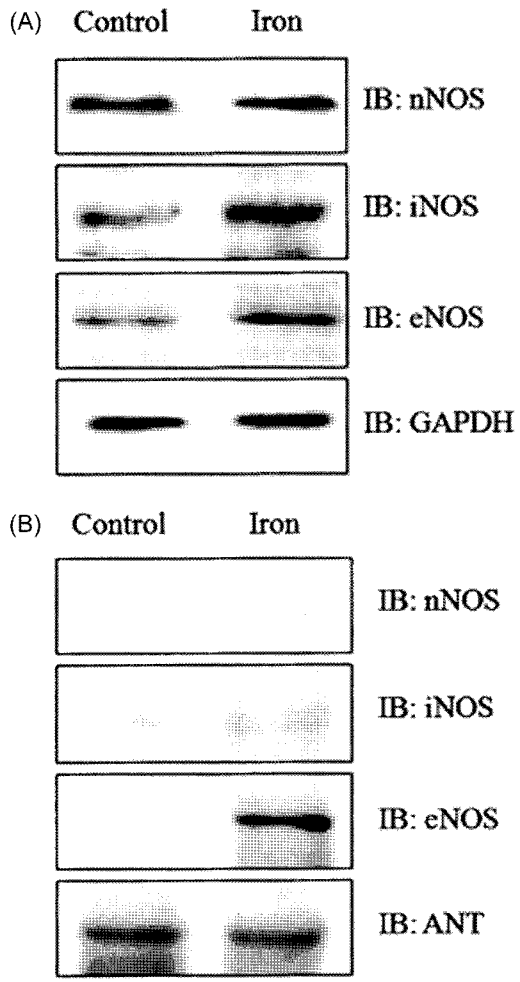

Fig. 4. Western blotting of NOS in lung tissue. Antibodies specific against iNOS, nNOS, and eNOS were used in western blotting of the tissue homogenate (A) and mitochondrial fraction (B). Lung homogenates or mitochondrial fraction were separated on 8% polyacrylamide gels for western blotting for NOS isozymes. Glyceraldehyde 3-phosphate dehydrogenase and adenine nucleotide translocator were used as a control for homogenate and mitochondria, respectively.

iron-overloaded lung, all three isoforms, namely, iNOS, eNOS, and nNOS, were analyzed by western blotting. In tissue homogenates, the levels of eNOS and iNOS were elevated (Fig. 4A). In fact, the increase in eNOS was less than that of iNOS due to iron. In the lung, high oxygen levels surrounding the alveolar cells require NO to protect them from oxidative damage. This unique environment may require constitutive eNOS expression similar to endothelial cells of blood vessels. Inducible iNOS can provide additional NO production to eliminate excess iron. Most significantly, eNOS was greatly enhanced in the mitochondria of the iron-overloaded lung (Fig. 4B). Previous studies have demonstrated NOS in brain mitochondria, which is similar to eNOS type (Lacza et al., 2001). Expression of NOS in the mitochondria and cytoplasm can be attributed to NO production due to iron accumulation in these high capacity sites.

Decreased RCR and cytochrome c oxidase activity following iron overload in the lung

Oxygen-associated damage in the lung can be observed early as mitochondrial impairment (O'Connell et al., 1991). Since the respiratory control ratio (RCR) is used typically to determine mitochondrial integrity and coupling of oxidative phosphorylation, we used this index to compare mitochondria from different iron-overloaded tissues in the rat. The RCR in lung mitochondria decreased 47% with little change found in the liver (Table 2). This reduction in the RCR was most likely a result of the 132% increase in the level of state 4 in the lung. A similar decrease of RCR was found in iron-overloaded liver mitochondria, but the extent of reduction was much less (22% versus 47%). The increase in the level of state 4 in the liver (increase by 22%) was less than that in lung. The levels of state 3 changed in

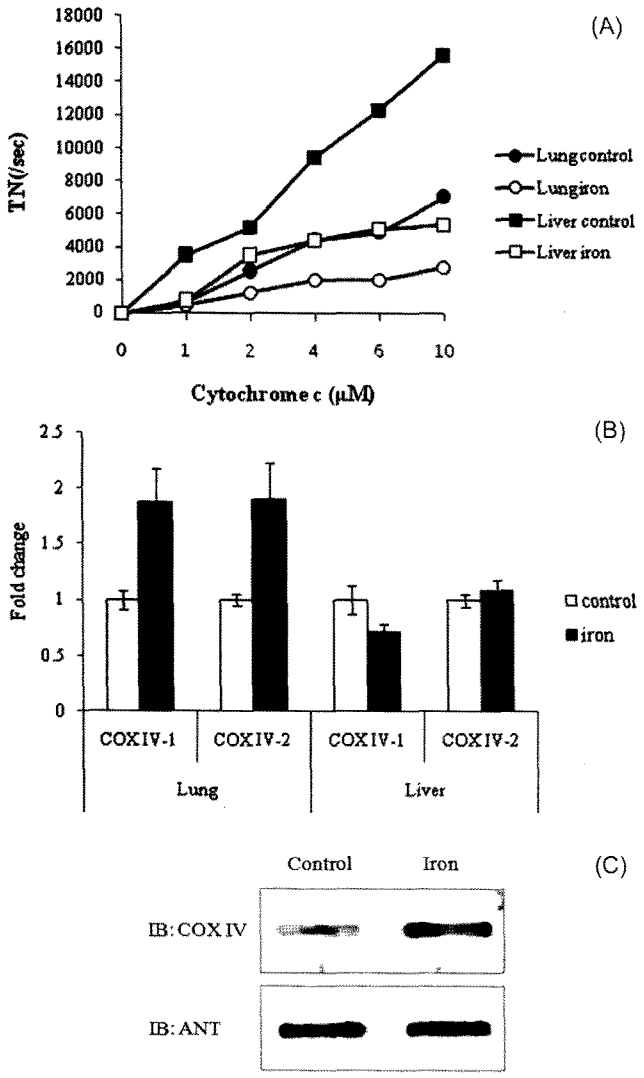

Fig. 5. Cytochrome c oxidase (CcO) activity and CcO subunit IV expression in the iron-overloaded lung tissue. (A) For CcO activity, mitochondria (up to 0.1 μM of heme aa_3) in 50 mM K-Pi (pH 7.4) and 1% Tween 20 were incubated with various concentration of cytochrome c in an oxygen electrode chamber at 25°C. (B) For RT-PCR of COX IV-1 and IV-2, total RNA was prepared using TRIZOL. To quantify COX IV-1 (isoform 1) and COX IV-2 (isoform 2) gene expression, real-time PCR (RT-PCR) was performed with citrate synthase as a control. (C) Immunoblot of CcO subunit IV with anti-CcO IV antibodies (MitoScience, Eugene, OR, USA) at a 1:1,000 dilution was carried out.

an opposing manner in the lung and liver. Whereas the lung exhibited a 23% increase, the liver demonstrated a 4% decrease. The high level of state 4 observed in mitochondria from the iron-overloaded lung may be caused by membrane damage via lipid peroxidation due to an inefficient antioxidant system or uncoupling relieved from ROS stress (Nègre-Salvayre et al., 1997). As the center of systemic iron homeostasis, the liver accumulates a greater amount of iron than the lung as shown in Fig. 2. The slight change in RCR in the liver compared with that in the lung suggests that protection of mitochondria through iron sequestration

Table 2. Effect of iron overload on respiratory control ratio of lung and liver mitochondria

		State 3	State 4	RCR
Lung	Control	5.61±1.42	1.76±0.70	3.18±0.43
	Iron	6.91±2.62	4.09±2.13	1.69±0.56
Liver	Control	10.19±3.38	2.08±0.88	4.91±0.59
	Iron	9.83±2.79	2.55±0.70	3.85±0.35

After addition of succinate (5 mM) and ADP (1 mM) to mitochondrial suspension (1 mg/mL) at 25°C in an oxygen electrode chamber, oxygen consumption was measured for state 3 until oxygen consumption rate was dropped for state 4.

by ferritin may be more efficient in the liver.

CcO activity with cytochrome c as the substrate was also reduced to 50% following iron overload in the lung and liver (Fig. 5A). Oxygen can be a limiting factor for CcO activity within these tissues since NO also competes with oxygen for CcO binding. Similar decrease in complex IV due to accumulated iron has been observed in Parkinson's disease (Harley et al., 1993).

Elevated expression of CcO subunits IV-1 and IV-2 in the iron-overloaded lung

In yeast mitochondria, the COX V subunit, which is a homolog of mammalian COX IV, isoform type 2 is expressed actively under hypoxic conditions with high kinetic activity (Waterland et al., 1991). A similar finding was reported with mammalian COX IV in which subunit IV-2 expression was sensitive to oxygen levels (Hüttemann et al., 2007). Therefore, to assess whether excess iron in the lung affects the expression of these two isoforms of COX IV, RT-PCR was performed. The mRNA levels of both isoforms exhibited approximately a 2-fold increase in the lung (Fig. 5B) with parallel increase of subunit IV (Fig. 5C). In the liver, however, the changes were not significant for either isoform.

DISCUSSION

Throughout an individual's life, the lung faces high oxygen pressure. Just as ROS are a powerful cause of cancer and aging, similar oxidative damage may induce widespread lung carcinoma. The vital role of mitochondria in iron metabolism, as well as in ROS production, led us to study iron related change in lung mitochondria. Increased ROS production parallel with iron content confirmed the causative role of iron in ROS production. ROS have been attributed to lung pathogenesis (Thannickal and Fanburg, 2000), which may be associated with mitochondrial dysfunction.

Because of its modulating role in iron and oxygen levels, NO level and NOS expression have been examined. NO in mitochondria was increased markedly along with the levels

of eNOS and iNOS in cells (Fig. 3 and 4A). Significantly, there was substantial expression of eNOS in isolated mitochondria (Fig. 4B) similar to that previously reported by Barnes and Belvisi (1993). A recent report demonstrated that mitochondrial NOS (mtNOS) binds the Vb subunit of CcO (Persichini et al., 2005). Direct contact of mtNOS with CcO most likely induces immediate effects as local NO inhibits the enzyme specifically as in Fig. 5A, leading to reduced respiratory activity (Chénais et al., 2002; Ramachandran et al., 2004). As a modulator of reactive oxygen, NO produced by mtNOS may perform a protective role under high oxygen pressure (Cooper, 2002; Haynes et al., 2003). However, explosive production of NO from excessive iron can inhibit CcO irreversibly, resulting in detrimental effects (Brown, 1999), such as oxygen conversion to superoxide by complex I (Galkin and Brandt, 2005) and/or complex III (Turrens et al., 1985). The increase in ROS demonstrated in this study supports this hypothesis. Despite the similar increase in ROS, RCR was much lower in lung mitochondria than in the liver. Mitochondria may be more susceptible to oxidative damage in the lung than in the liver; however, the cause of this variation is presently unclear. Thus, the superoxide with NO would generate peroxynitrite, which is a potent reactive nitrogen species (RNS). Even though CcO plays a vital role in the metabolism of NO (Liu et al., 2004; Castello et al., 2006, 2008) and peroxynitrite (Pearce et al., 2002), the enzyme can be impaired itself during the process. Too much peroxynitrite can destroy the heme-containing subunit I, a major catalytic subunit of CcO (Sharpe and Cooper, 1998), leading to irreversible inactivation of enzyme and resultant low enzyme activity. Taken together, these observations demonstrate that, while NO may generally be protective, overproduction in the presence of superoxide could be harmful due to peroxynitrite production that irreversibly alters CcO with increased Km (Cooper et al., 2003; Cooper and Giulivi, 2007). Thus, vast amounts of NO with superoxide could be as deleterious as ROS and RNS in causing irreversible inhibition of CcO and respiratory activity (Brunori et al., 2006). Once altered, CcO can cause these harmful compounds to accumulate, thereby further accelerating this deleterious cycle.

As NO is thought to decrease oxygen pressure, hypoxic conditions likely occur during iron overload. Because isoform Vb is associated with NO and hypoxia in yeast, we examined whether the hypoxic isoform IV-2 was also overproduced in our iron overload conditions. Subunit V of yeast, which is the mammalian homologue of subunit IV, exists as different isoforms depending on the availability of oxygen (Trueblood and Poyton, 1987). For example, the Vb isozyme, which is homologous to mammalian IV-2, is expressed under hypoxic conditions with a high kinetic constant (Waterland et al., 1991). Later studies also revealed similar isoforms in cancerous lung tissue (Hüttemann et al., 2001). Similar to Vb, the mammalian subunit IV-2 contains an oxygen responsive element (Hüttemann et al., 2007), suggesting that isoforms of this subunit may possess enzymatic activity that is affected by oxygen pressure, possibly in the presence of NO. Intriguingly, we found parallel increases of both isoforms (Fig. 5B). The tissue-specific isoform, which is expressed in epithelial cells or smooth muscle cells of the lung, may be responsible for these increases as expression of subunit IV-2 has been reported to be particularly augmented in cortical astrocytes under hypoxia (Horvat et al., 2006) and in smooth muscle cells in human lung cancer (Hüttemann et al., 2001). However, in the liver, expression of these isoforms did not change significantly, similar to the differential response of CcO to iron (Vijayasarathy et al., 1998). Alternatively, degraded CcO due to subunit I destruction may demand nascent production of enzyme subunits. As subunit IV is necessary for the initial assembly of CcO, its new synthesis, irrespective of isoform, may be required for the biogenesis of the enzyme (Li et al., 2006).

In this study, we demonstrate that iron overload in the lung increased ROS, NO, NOS protein, as well as expression of CcO subunit IV, while decreasing CcO activity and RCR. Most significantly NOS in mitochondria (mtNOS) was highly expressed. These findings show that elevated NO production and diminished CcO activity occur along with high ROS and low RCR, as expected. These results suggest that CcO inhibition is associated with NO production during iron overload. If the lung relies on CcO activity for NO metabolism, damaged CcO should be detrimental to the cell due to uncontrolled production of ROS and RNS (Pearce et al., 2002). We propose that iron-induced NO and the associated CcO inhibition via ROS and RNS are the leading causes of lung pathology in diseases related to iron overload.

ACKNOWLEDGMENTS

This work was supported by Sookmyung Women's University for 2007.

REFERENCES

Arnold S and Kadenbach B (1997) Cell respiration is controlled by ATP, an allosteric inhibitor of cytochrome-c oxidase. *Eur J Biochem* 249: 350-354.

Barnes PJ and Belvisi MG (1993) Nitric oxide and lung disease. *Thorax* 48: 1034-1043.

Brown GC (1999) Nitric oxide and mitochondrial respiration. *Biochim Biophys Acta* 1411: 351-369.

Brown GC and Cooper CE (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* 356: 295-298.

Brunori M, Forte E, Arese M, Mastronicola D, Giuffrè A, and Sarti P (2006) Nitric oxide and the respiratory enzyme. *Biochim Biophys Acta* 1757: 1144-1154.

Carnelli V, D'Angelo E, Pecchiari M, Ligorio M, and D'Angelo E (2003) Pulmonary dysfunction in transfusion-dependent patients with thalassemia major. *Am J Respir Crit Care Med* 168: 180-184.

Castello PR, David PS, McClure T, Crook Z, and Poyton RO (2006) Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab* 3: 277-287.

Castello PR, Woo DK, Ball K, Wojcik J, Liu L, and Poyton RO (2008) Oxygen-regulated isoforms of cytochrome c oxidase have differential effects on its nitric oxide production and on hypoxic signaling. *Proc Natl Acad Sci USA* 105: 8203-8208.

Chénais B, Morjani H, and Drapier JC (2002) Impact of endogenous nitric oxide on microglial cell energy metabolism and labile iron pool. *J Neurochem* 81: 615-623.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

Claireaux AE (1975) Drugs and the lung. The effect of oxygen on the lung. *J clin Path 28 Suppl. (Roy Coll Path)* 9: 75-80.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, and Schapira AH (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett* 345: 50-54.

Cooper CE (2002) Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends Biochem Sci* 27: 33-39.

Cooper CE and Giulivi C (2007) Nitric oxide regulation of mitochondrial oxygen consumption II: Molecular mechanism and tissue physiology. *Am J Physiol Cell Physiol* 292: C1993-2003.

Cooper CE, Davies NA, Psychoulis M, Canevari L, Bates TE, Dobbie MS, Casley CS, and Sharpe MA (2003) Nitric oxide and peroxynitrite cause irreversible increases in the K(m) for oxygen of mitochondrial cytochrome oxidase: in vitro and in vivo studies. *Biochim Biophys Acta* 1607: 27-34.

Eastabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratio. *Methods Enzymol* 10: 41-47.

Factor JM, Pottipati SR, Rappoport I, Rosner IK, Lesser ML, and Giardina PJ (1994) Pulmonary function abnormalities in thalassemia major and the role of iron overload. *Am J Respir Crit Care Med* 149: 1570-1574.

Galkin A and Brandt U (2005) Superoxide radical formation by pure complex I (NADH:Ubiquinone Oxidoreductase) from *Yarrowia lipolytica*. *J Biol Chem* 280: 30129-30135.

Galleano M, Simontacchi M, and Puntarulo S (2004) Nitric oxide and iron: effect of iron overload on nitric oxide production in endotoxemia. *Mol Aspects Med* 25: 141-154.

Gutteridge JM, Quinlan GJ, and Evans TW (2001) The iron paradox of heart and lungs and its implications for acute lung injury. *Free Radic Res* 34: 439-443.

Harley A, Cooper JM, and Schapira AH (1993) Iron induced oxidative stress and mitochondrial dysfunction: relevance to Parkinson's disease. *Brain Res* 12: 349-353.

Haynes V, Elfering SL, Squires RJ, Traaseth N, Solien J, Ettl A, and Giulivi C (2003) Mitochondrial nitric-oxide synthase: role in pathophysiology. *IUBMB Life* 55: 599-603.

Horvat S, Beyer C, and Arnold S (2006) Effect of hypoxia on the transcription pattern of subunit isoforms and the kinetics of cytochrome c oxidase in cortical astrocytes and cerebellar neurons. *J Neurochem* 99:937-951.

Hüttemann M, Kadenbach B, and Grossman LI (2001) Mammalian subunit IV isoforms of cytochrome c oxidase. *Gene* 267: 111-123.

Hüttemann M, Lee I, Liu J, and Grossman LI (2007) Transcription of mammalian cytochrome c oxidase subunit IV-2 is controlled by a novel conserved oxygen responsive element. *FEBS J* 274: 5737-5748.

Kakhlon O and Cabantchik ZI (2002) The labile iron pool: characterization, measurement, and participation in cellular processes. *Free Radic Biol Med* 33: 1037-1046.

Kamp DW, Panduri V, Weitzman SA, and Chandel N (2002) Asbestos-induced alveolar epithelial cell apoptosis: role of mitochondrial dysfunction caused by iron-derived free radicals. *Mol Cell Biochem* 234-235: 153-160.

Kolb JP, Paul-Eugene N, Damais C, Yamaoka K, Drapier JC, and Dugas B (1994) Interleukin-4 stimulates cGMP production by IFN-gamma-activated human monocytes. Involvement of the nitric oxide synthase pathway. *J Biol Chem* 269: 9811-9816.

Lacza Z, Puskar M, Figueroa JP, Zhang J, Rajapakse N, and Busija DW (2001) Mitochondrial nitric oxide synthase is constitutively active and is functionally upregulated in hypoxia. *Free Rad Biol Med* 31: 1609-1615.

Lancaster Jr JR and Hibbs Jr JB (1990) EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc Natl Acad Sci USA* 87: 1223-1227.

Levi S, Luzzago A, Cesareni G, Cozzi A, Franceschinelli F, Albertini A, and Arosio P (1988) Mechanism of ferritin iron uptake: activity of the H-chain and deletion mapping of the ferro-oxidase site. A study of iron uptake and ferro-oxidase activity of human liver, recombinant H-chain ferritins, and of two H-chain deletion mutants. *J Biol Chem* 263: 18086-18092.

Li Y, Park JS, Deng JH, and Bai Y (2006) Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex. *J Bioenerg Biomembr* 38: 283-291.

Liu X, Cheng C, Zorko N, Cronin S, Chen YR, and Zweier JL (2004) Biphasic modulation of vascular nitric oxide catabolism by oxygen. *Am J Physiol Heart Circ Physiol* 287: H2421-2426.

Napier I, Ponka P, and Richardson DR (2005) Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood* 105: 1867-1874.

Nègre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, Pénicaud L, and Casteilla L (1997) A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J* 11: 809-815.

Nestel FP, Greene RN, Kichian K, Ponka P, and Lapp WS (2000) Activation of macrophage cytostatic effector mechanisms during acute graft-versus-host disease: release of intracellular iron and nitric oxide-mediated cytostasis. *Blood* 96: 1836-1843.

O'Connell MJ, Snape SD, and Nunn JF (1991) An early marker of hyperoxic lung injury in the rat and its pharmacological modulation. *Br J Anaesth* 66: 697-702.

Pande SV and Blanchaer MC (1971) Reversible inhibition of mitochondria adenosine diphosphate phosphorylation by long chain acyl coenzyme A esters. *J Biol Chem* 25: 402-411.

Pearce LL, Kanai AJ, Birder LA, Pitt BR, and Peterson J (2002) The catabolic fate of nitric oxide: the nitric oxide oxidase and peroxynitrite reductase activities of cytochrome oxidase. *J Biol Chem* 277: 13556-13562.

Persichini T, Mazzone V, Polticelli F, Moreno S, Venturini G, Clementi E, and Colasanti M (2005) Mitochondrial type I nitric oxide synthase physically interacts with cytochrome c oxidase. *Neurosci Lett* 384: 254-259.

Ramachandran A, Ceaser E, and Darley-Usmar VM (2004) Chronic exposure to nitric oxide alters the free iron pool in endothelial cells: role of mitochondrial respiratory complexes and heat shock proteins. *Proc Natl Acad Sci USA* 101: 384-389.

Sharpe MA and Cooper CE (1998) Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. *J Biol Chem* 273: 30961-30972.

Thannickal VJ and Fanburg BL (2000) Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005-1028.

Trueblood CE and Poyton RO (1987) Differential regulation of the two genes encoding Saccharomyces cerevisiae cytochrome c oxidase subunit V by heme and the HAP2 and REO1 genes. *Mol Cell Biol* 7:3520-3526.

Turrens JF, Alexandre A, and Lehninger AL (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237: 408-414.

Valkonen M and Kuusi T (1997) Spectrophotometric assay for total peroxyl radical-trapping antioxidant potential in human serum. *J Lipid Res* 38: 823-833.

Vijayasarathy C, Biunno I, Lenka N, Yang M, Basu A, Hall IP, and Avadhani NG (1998) Variations in the subunit content and catalytic activity of the cytochrome c oxidase complex from different tissues and different cardiac compartments. *Biochim Biophys Acta* 371: 71-82.

Waterland RA, Basu A, Chance B, and Poyton RO (1991) The isoforms of yeast cytochrome c oxidase subunit V alter the in vivo kinetic properties of the holoenzyme. *J Biol Chem* 266: 4180-4186.

[Received May 18, 2009; accepted June 22, 2009]