Original Article

ISSN: 1735-0344 *Tanaffos 2012; 11(4): 23-29*

TANAFFOS

Reduced Denitration Activity in Peripheral Lung of Chronic Obstructive Pulmonary Disease

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Received: 2 December 2012
Accepted: 15 December 2012

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Background: Accumulation of nitrated protein is seen in peripheral lung and cells from patients with chronic obstructive pulmonary disease (COPD). Nitrated protein causes abnormal protein function, but the nitration was believed to be an irreversible process. However, there are accumulating evidences that this process is reversible by an active denitration pathway. The aim of this study is to detect denitration activity in protein extracts from peripheral lung tissue of COPD and to compare with those in healthy subjects.

Materials and Methods: Peripheral lung tissue from 4 healthy, 4 smokers without COPD, 4 GOLD stage 1 and 4 GOLD stage 2 were used for denitration assay. Denitration activity was determined as reduction of nitro-tyrosine level of nitrated histone protein after incubation with protein extracts from peripheral lung, which was determined by western blotting. In addition, RNA is extracted from peripheral lung of 8 healthy, 7 smoking control, 8 stage 1 and 2 COPD and 10 stage 3 and 4 COPD and nitrate reductase mRNA expression was determined by real time RT-PCR.

Results: Peripheral lung protein extracts from healthy subjects reduced nitro-tyrosine level of nitrated histone. Thus, we were able to show denitration activity in peripheral lungs. The denitration activity was slightly reduced in smoking controls, and significantly reduced in COPD patients. We also showed that the expression of the human homologue of nitrate reductase (chytochrome β2 reductase), a potential candidate of denitrase, was significanty reduced in COPD lung.

Conclusion: This study suggests that accumulation of nitrated protein in lung tissue of COPD may, at least in part, be induced by a reduction in denitration activity or nitrate reductase.

Key words: COPD, Nitrotyrosine, Denitration, Nitrate reductase, Nitrative stress

INTRODUCTION

Peroxynitrite is a potent reactive nitrogen species formed by the rapid interaction of superoxide radical ($O_2^{\cdot-}$) and nitric oxide (NO) (1), and the level was elevated in exhaled breath condensate in patients with chronic obstructive pulmonary disease (COPD)(2). Protein nitration is one of the well-studied effects of peroxynitrite[1] and it leads to the formation of 3-nitrotyrosine residues (3). Tyrosine nitration has been reported to cause protein dysfunction and/or degradation (4,5). For example, manganese superoxide dismutase (MnSOD) has been reported to be inactivated due to protein tyrosine nitration (6), resulting in a further enhancement of the nitrative/oxidative stress burden. The proteosome has also been reported to be inactivated when exposed to peroxynitrite (7), suggesting that levels of degradation are also affected by oxidative/nitrative stress. Clinically, formation of nitrotyrosine residues is used as a

pathological marker and has been reported in a range of diseases such as Parkinson's disease, diabetes and chronic airway disease (4, 8, 9). Due to the pathological effects of protein tyrosine nitration, mechanisms such as proteolytic degradation of tyrosine nitrated proteins have been suggested as being important in removing these 'abnormal' proteins from the cell (5).

Protein nitration has been thought of as an irreversible process although there have been reports of denitration systems by nitroreductase or other similar enzymes in non-mammalian species (10-13). In addition, several reports have described a repair mechanism for nitrated proteins, called denitration activity. The activity has been reported to reduce the nitrotyrosine immunoreactivity of nitrated bovine serum albumin (BSA) or nitrated histone in homogenates from rat spleen and lung (14), rat brains and heart(15), dog prostrate (16) and a murine cell line (17). This activity was also inducible by LPS in a murine cell line (17). However, the activity has not previously been detected in human cells nor its expression investigated in disease. We therefore aimed to determine denitration activity in human lung tissue from non-smokers, smoking controls and COPD.

MATERIALS AND METHODS

Material

Bradford assay kit was obtained from Bio-Rad laboratories (Hemel Hempstead, UK); mouse monoclonal anti-nitrotyrosine antibody from Millipore Ltd (Watford, UK); horse-radish-peroxidase HRP-conjugate anti-mouse secondary antibodies from DakoCytomation, (Cambridgeshire, UK). Histone protein (Sigma Ltd Poole, UK) was nitrated (200 mM $NaHCO_3$, 500 µM 3-Morpholinosydnonimine.HCl (SIN-1) and precipitated in the presence of cold acetone as previously reported (18).

Preparation of protein extracts from peripheral lung

COPD disease severity was graded a*ccording to the GOLD guidelines (19)* with lung function and symptoms. Lung tissue were obtained from an established tissue bank (20, 21) linked to an established patient registry. The baseline characteristics of the patients are summarized in Table 1 and 2.

Table 1. Demography of the subjects used for denitraion activity assay

	Healthy	Smoker w/o COPD	COPD GOLD 1	COPD GOLD 2
N	4	4	4	4
Age	59 ± 6.1	58 ± 3.2	62 ± 5.9	62 ± 7.7
Male/Female	2/2	3/1	3/1	2/2
FEV1 post (predicted%)	101 ± 8.4	94.8 ± 1.9	85.5 ± 1.6	69.3 ± 3.9
FEV1/FVC (predicted%)	82.8 ± 1.5	78.0 ± 3.1	62.8 ± 2.8	63.5 ± 1.6
KCO (predicted %)	84.8 ± 7.1	78.5 ± 8.3	72.0 ± 16.5	78.8 ± 15.4
Pack/year	0 ± 0	32.8 ± 6.3	41.5 ± 25	53.8 ± 12.8

FEV_1: forces expiratory volume in 1 s;
KCO: Carbon monoxide transfer coefficient

Table 2. Demography of the subjects used for NARD mRNA detection

	Healthy	Smoker w/o COPD	COPD GOLD 1&2	COPD GOLD 3&4
N	8	7	8	10
Age	57 ± 6.5	62 ± 3.5	57 ± 3.3	60 ± 2.1
Male/Female	4/4	4/3	3/1	2/2
FEV1 post (predicted %)	99.7 ± 5.1	103.5 ± 3.8	68.9 ± 2.7	26.7 ± 3.6
FEV1/FVC (predicted %)	81.3 ± 1.3	75.7 ± 1.9	58.3 ± 2.3	41.0 ± 3.8
KCO (predicted %)	78.5 ± 3.2	72.3 ± 2.7	65.1 ± 5.0	52.3 ± 7.3
Pack/year	0 ± 0	49.5 ± 10.3	40.0 ± 8.0	52.0 ± 5.9

FEV_1: forces expiratory volume in 1 s;
KCO: Carbon monoxide transfer coefficient

Peripheral lung tissues specimens (three pieces approximately 0.5 cm by 0.5 cm by 0.5 cm in size) from non-smokers, smokers and COPD patients at stages 1 and 2 (mild and moderate COPD, respectively) were ground under liquid nitrogen using a pestle and mortar. Hypotonic buffer [10 mM HEPES- sodium hydroxide, pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM 2-mercaptoethanol and one protease inhibitor cocktail tablet per 10 mL (Roches Diagnostics, Lewes, UK)] was added to samples to remove containing red cells and secretions and to loosen cell membranes, and then left for 15 min on ice.

The samples were re-suspended in protein extraction buffer (50mM TrisHCl pH 7.4, 500 mM NaCl with a protein inhibitor tablet) and left for 20 min on ice, then sonicated with vibra-cell high density ultrasonic processor (Jencons, Bedfordshire, UK) for 10 sec with amplitude 60. Samples were then centrifuged again at 12000 rpm for 10 min at 4°C. Immediately the supernatant was transferred into fresh cold labelled microcentrifuge tubes and the protein concentration of the cell lysate determined with Bradford Bio-Rad Protein Assay kit using bovine serum albumin as a standard (Biorad, Hemel Hempstead, UK).

Denitration activity in COPD peripheral lung tissue

Protein extracts from peripheral lung tissue were incubated with nitrated histone (20 µl of 25 µg/ml) for 3 hr at 37°C in the presence of the proteasome inhibitor ALLN (50 µM). Protein separation was carried out on SDS-PAGE followed by immunoblot analysis using Xcell SuperLock Mini-Cell and Immunoblot apparatus (Invitrogen Ltd (Paisley, UK)). Nitrotyrosine adducts on nitrated histone were detected using a mouse monoclonal anti-nitrotyrosine antibody and the densities of western blotting band visualized by enhanced chemiluminescent (ECL) developing solution on Hybond ECL nitrocellulose membrane (Amersham Biosciences (Bukinghamshire, UK)) were determined by densitometric analysis.

The total denitration activity was calculated and shown as % reduction of nitrotyrosine residues on nitrated Histone (nHist) which is not incubated with lung tissue extracts (basal is either 100% or 0%) as shown in the formula:

Total Denitration activity =

100 x [1- (nitrotyrosine on Histone in the presence of peripheral lung extracts)/(nitrotyrosine on Histone in the absence of extracts)]

Expression of nitrate reductase (NARD) in COPD peripheral lung tissue

Following the manufacture's instructions from the RNeasy kit (Qiagen), RNA was extracted from human lung tissue; age-matched non-smokers (n=8), healthy smokers (n=7), patients with mild and moderate COPD (stages 1 and 2 respectively, n=8), severe and very severe COPD (stages 3 and 4 respectively, n=10), and converted to cDNA using Omniscript RT (Qiagen). cDNA was then subjected to real-time quantitative polymerase chain reaction (RT-QPCR) analysis using Taqman master mix and primers (Applied Biosystems (California, USA)) in Rotor-Gene 3000 from Corbett Research (Sydney, Australia). The relative copy number of mRNA of NADH cytochrome β-5 reductase (CYB5R3) (the human homologue of nitrate reductase (hNARD) was determined using standard curve of the house keeping gene, guanine nucleotide binding protein beta polypeptide-2-like 1 (GNB2L1), and then normalized to the copy number of house keeping gene GNB2L1.

RESULTS

Denitration activity in COPD peripheral lung tissue

Peripheral lung tissue from non-smokers (NS) decreased nitrotyrosine residues on nitrated histone (nHistone) to 19.2±2.6% of nHistone control as shown in Figure 1 A & B, which represents total denitration activity (80.8% denitration activity). In contrast, denitration activity was significantly reduced in peripheral lung tissue in healthy smokers (53.0±5.2% vs. 80.8±2.6% in NS) and further reduced with increasing severity of COPD (stage 1 (31.3±4.8%) and stage 2 (17.6±3.0%) vs. 80.8±2.6% non-smokers and 53.0±5.2% healthy smokers) (Figure 1).

Expression of nitrate reductase (NARD) in COPD peripheral lung tissue

The mRNA level of nitrate reductase (human homolog: cytochrome β5 reductase) in smokers without COPD

showed the trend of reduction when compared with normal subjects, but not significant (Figure 2). However there was a significant reduction mRNA of NARD in peripheral lung of COPD patients when compared with normal subjects [non-smokers 0.419±0.05; smokers 0.296±0.04; mild and moderate COPD (stages 1 and 2) 0.235±0.02; severe and very severe COPD (stages 3 and 4) as seen in Figure 2. There are good correlations between mRNA levels of NARD and lung functions (A: FEV_1, B: FEV_1/FVC, C:KCO in Figure 3) , but no correlation with smoking history and age was detected.

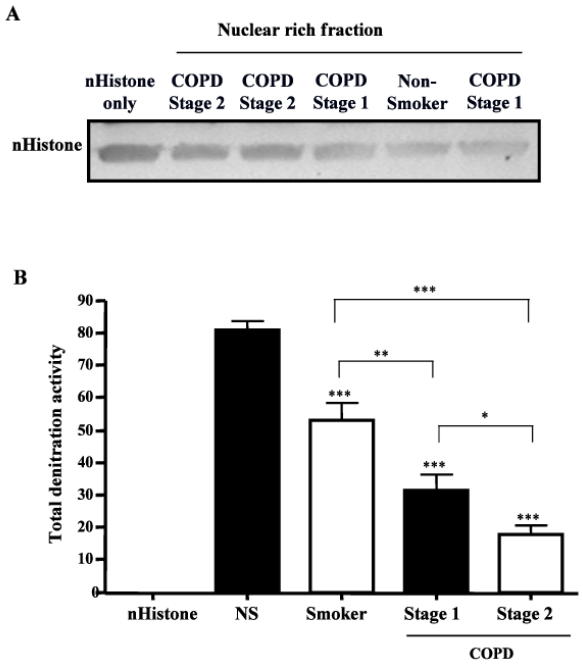

Figure 1 Denitration activity in COPD peripheral lung tissue. Protein extracts from peripheral lung tissue was incubated with nitrated histone (nHistone) for 3 hr at 37°C in the presence of the proteasome inhibitor ALLN (50 μM). A representative Western blot image of nHistone is shown in (A). Quantification of the Western blot data by densitometric analysis is shown in (B) and denitration activity was presented as mean ± SEM. NS, non-smoker; COPD, chronic obstructive pulmonary disorder; NS, n = 4; Smoker, n = 4; Stage 1 (mild COPD), n = 4; Stage 2 (moderate COPD), n = 4; *** $p<0.001$ vs non-smoker, ** $p<0.01$, * $p<0.05$.

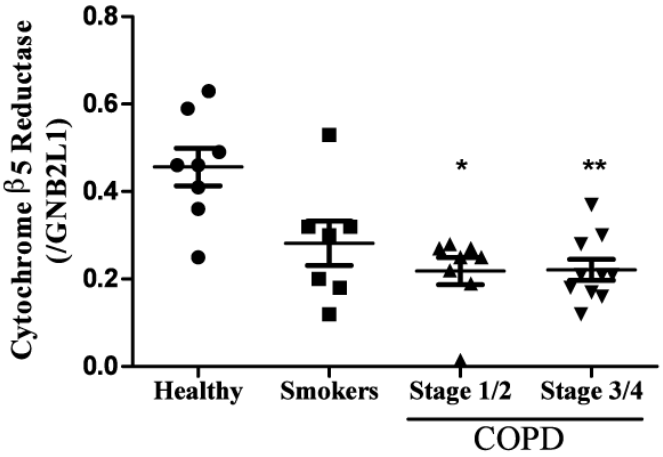

Figure 2 Nitrate reductase expression in COPD peripheral lung tissue. Nitrate reductase mRNA was extracted from age-matched non-smokers (n=8), healthy smokers (n=7), patients with mild and moderate COPD (stages 1 and 2 respectively, n=8) and severe and very severe COPD (stages 3 and 4 respectively, n=10). ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$ vs non-smoker.

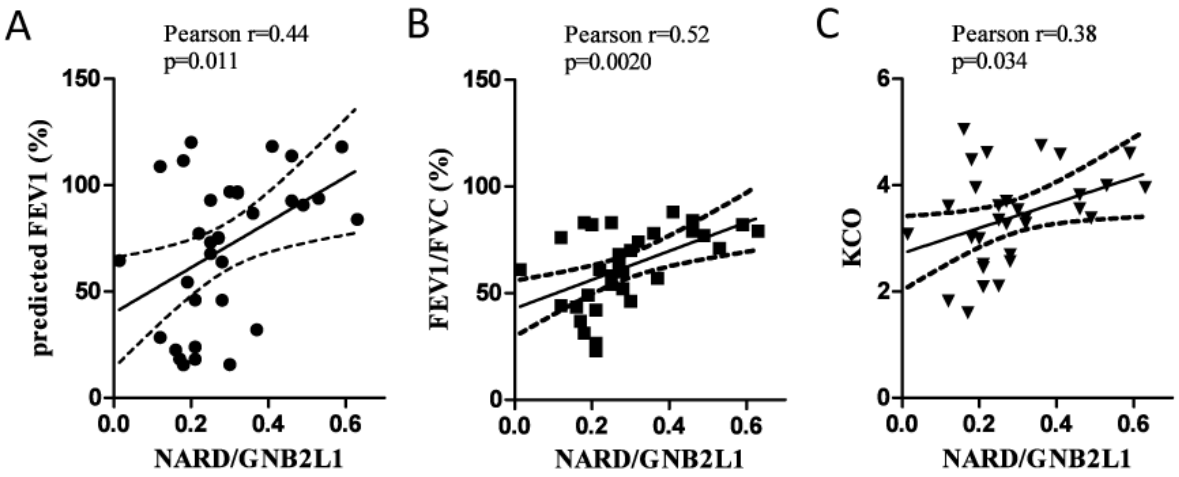

Figure 3 Relationship between Nitrate reductase expression and lung function Nitrate reductase mRNA was determined by RT-PCR and correlation with FEV1 predicted % (A), FEV1/FVC ratio (B) and KCO predicted % (C). Samples were collected from age-matched non-smokers (n=8), healthy smokers (n=7), patients with mild and moderate COPD (stages 1 and 2 respectively, n=8) and severe and very severe COPD (stages 3 and 4 respectively, n=10). Peasrson r value and p value were shown in graph.

DISCUSSION

Protein nitration was originally believed to be an irreversible process but a denitration activity by a putative enzyme, denitrase, has recently been demonstrated in homogenised extracts from rat spleen and lung (14), rat brain and heart (15), dog prostrates (16) and a mouse cell line (17, 22). In this manuscript, for the first time, denitrase or denitration activity was confirmed in human peripheral lung tissue. Importantly, denitration activity was reduced in peripheral lung samples of patients with increasing severity of COPD.

There are a several reports showing the accumulation of nitrated proteins in lung tissue and sputum cells (9, 23-25) and skeletal muscle (26). Tyrosine nitration has been reported to alter both protein function and breakdown (3, 27-29). Histone deacetylase 2 (HDAC2) is also nitrated and the level nitration affects its expression and activity (29), causing amplified inflammation and corticosteroid insensitivity seen in patients with COPD (18, 20). The accumulation of nitrated proteins might result from (1) an increased oxidative stress/nitrative burden in COPD compared with smokers(30), (2) a reduction in the anti-oxidant capacity in COPD (31) or (3) a reduction in proteasome function in COPD(32), resulting in a reduction in the breakdown of nitrated proteins. In this study, an alternative hypothesis, namely that the increase in tyrosine nitrated proteins seen in COPD is as a result of a reduction in an active denitration process, has been examined. As seen in Figure 1, we confirmed reduction of denitration activity in COPD peripheral lung.

Kamisaki and colleagues demonstrated that denitration activity was trypsin sensitive (14, 15, 17). With the evidence combined, denitration activity is catalysed by a novel protein-like factor, probably a true enzyme in nature, referred to as 'denitrase' in this manuscript and as reported previously. The enzyme responsible for denitration activity has not been identified. Kamisaki and colleagues demonstrated that a protein less than 10 KDa had denitration activity (14). In addition, Kuo showed that *E.coli* enzymes; nitroreductase (NORD) and nitrate reductase (NARD) possessed denitration activity (33). Kalns and colleagues also demonstrated that nitrate reductase altered 3-nitrotyrosine accumulation and cell cycle progression in LPS + IFN-gamma-stimulated RAW 264.7 cells (22). NARD is a very efficient enzyme present in plants for nitrogen metabolism. In plants, NARD uses

cofactors such as NADH to reduce nitrate (NO_3^-) to nitrite (NO_2^-) and then finally to ammonium ions (NH_4^+) which plants can easily utilise. Kuo and colleagues speculate that nitrotyrosine is structurally quite similar with nitrate because nitrotyrosine (Tyr-NO_2) has a tyrosine residue instead of one oxygen molecule in NO_3^- ($^-O=NO_2$), suggesting that NARD can target nitrotyrosine. Previous reports have confirmed the release of nitrate during incubation of mixture of NARD and nitrated protein (15, 33). The human homolog of the enzyme, *E.coli* nitrate reductase (NARD) was chytochrome β2 reductase. As shown in Fig.2, the mRNA level of nitrate reductase was reduced in peripheral lung tissue from COPD patients when normalized to the oxidative stress resistant house keeping gene, guanine nucleotide binding protein beta polypeptide-2-like 1 (GNB2LB), which is well correlated with the level of denitration activity. The NARD levels were correlated well with FEV1, FEV1/FVC ratio and KCO, but not with age and smoking history (pack year).

Thus, denitration activity was found first time in human peripheral lung, and reduced denitration activity found in peripheral lung will cause accumulation of nitrated protein, leading abnormal cell function. Therefore, enhancing denitration activity might be an attractive approach to reduce inflammation and restore corticosteroid sensitivity in COPD and other diseases where high levels of nitrative stress are seen.

Acknowledgment

The current project supported by grant of Mitsubishi Pharma (current Mitsubishi-Tanabe Pharma, Japan).

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