行政院國家科學委員會專題研究計畫 期中進度報告

Tetrahydrobiopterin 對於 NO 合酶成之調控(2/3)
期中進度報告(精簡版)

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Introduction

The nitric oxide synthases (NOS) are responsible for the production of nitric oxide (NO) in different tissues and cells. To date, three distinct isoforms of NOS have been described [1]. nNOS is predominantly found in neuronal tissue, iNOS is inducible in a wide range of cells and tissues, and eNOS is the isoform in vascular endothelial cells. These isoforms have in the past been also differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression. NO synthesized in endothelial cells diffuses to vascular smooth muscle where it activates the cytosolic form of guanylate cyclase to modulate vascular smooth muscle tone [2]. In the nervous system, NO normally functions as a physiological neuronal mediator. In macrophage, iNOS is markedly stimulated by bacterial lipopolysaccharide, and NO combines with oxygen free radicals to form compounds that are more bactericidal than NO itself.

NOS is a dimeric enzyme composed of two catalytic domains: a C-terminal reductase domain, which binds NADPH, FMN, and FAD, and an N-terminal oxygenase domain, which binds a prosthetic heme group, BH_4, oxygen, and L-arginine [3]. The catalytic production of nitric oxide involves flavin-mediated electron transfer from C-terminal bound NADPH to the N-terminal heme center [3]. At the heme site, oxygen is reduced and incorporated into the guanidino group of L-arginine, producing nitric oxide (NO) and L-citrulline. BH_4 plays a critical role in allowing electron transfer from the prosthetic heme to L-arginine. In the absence of BH_4, electron flow from the reductase domain to the oxygenase domain is diverted to molecular oxygen rather than to L-arginine, leading to a condition known as NOS uncoupling, which causes production of superoxide rather than nitric oxide [4, 5]. Superoxide reacts rapidly with NO to form the peroxynitrite anion ($ONOO^-$), which is a strong biological oxidant known to oxidize lipids, protein, sulfhydryls, and DNA and to cause nitration of tyrosines.

However, overproduction of NO is highly toxic [6]. Excessive and prolonged activation of iNOS is associated with inflammatory tissue damage in various disease states, such as septicemia, rheumatoid arthritis, neurodegenerative diseases, and diabetes mellitus [7]. Thus, inhibition of excessive NO generation, particularly from iNOS, has been considered as one method to ameliorate inflammatory tissue injury [6]. Inhibition of tetrahydrobiopterin synthesis reduced nitric oxide synthesis by iNOS in rat treated by lipopolysaccharide (LPS) [8]. A multicentered, randomized, and placebo-controlled clinical trial has been conducted for the effects of the nitric oxide synthase inhibitor 546C88 in patients with septic shock, and the treatment could reduce the elevated plasma nitrate concentrations and was associated with an increase

in vascular tone [9]. But the trial was stopped early by the increased mortality in experimental group [10].

With these increasing, but still confusing, therapeutic consideration on the BH_4-NOS axis, we have explored the therapeutic effect of BH_4 by escalating its dosage. We have shown that mice treated lipopolysaccharides (LPS) showed elevation of nitrite, superoxide, and peroxynitrite. Very interestingly, the administration of BH_4 decreased the production of superoxide and peroxynitrite.

5-methyl-6,7,8-trihydrobiopterin (5-Me-BH4) can substitute BH4 as a cofactor . This BH4 analogue proved to be a valuable tool for studying the catalytic mechanism. First there were relevant to the continuing debate about whether BH4 is redox-active in NOS [3±5]. The mere observation of NO synthesis stimulated by 5-Me-BH4 ruled out a redox cycle between BH4 and quinonoid BH2 [6,7-dihydro(8*H*)biopterin] as an essential element in the catalytic mechanism, because 5-Me-BH4 cannot be reversibly oxidized to a quinonoid dihydro species [11]. However, it has also been shown that only fully reduced BH4-diminished superoxide release from eNOS, with efficiency BH4 > 6-methyl-BH4 > 5-methyl-BH4. In contrast, partially oxidized BH4 analogues, 7,8-dihydrobiopterin (7,8-BH2) and sepiapterin had no effect [12].

Bleomycin (BLM) is commonly used as a part of the cytostatic treatment of several tumor types, such as germ-cell tumors, lymphomas, and Kaposi's squamous cell carcinomas of head and neck. The application of BLM is featured by the occurrence of some fetal side effects. Pulmonary toxicity is the most serious side effect of BLM. In lung, the toxicity of BLM involved inflammation and fibrosis [13]. Previous study has suggested that the endogenous nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS) in lung, mediates pulmonary toxicity of BLM [14], and has demonstrated that aminoguanidine (AG), a preferred iNOS inhibitor, ameliorates the development of pulmonary injury and fibrosis induced by BLM [15]. It is further shown that endogenous peroxinitrite mediated BLM [16].

In the past few months, we first extended the measurement of NO, from measuring only nitrite to assaying both nitrite and nitrate. We then tested several of the modified compounds from BH4, that is, 5-methyl-tetrahydrobiopterin and 6-methyl-tetrahydrobiopterin. These compounds have slightly decreased catalytic activity, but also different in the liability to be oxidized.The other approach is to establish a mice bleomycin lung fibrosis model. The application of bleomycin will be through tracheostomy. We have practiced and learned the surgery, and now we can easily apply the drug to mice. Lung fibrosis and lung inflammation/free radical production were studied.

Materials and Methods

Chemicals 5.6.7.8-tetrahydrobiopterin (BH_4), 5-methyl- BH_4, and 6-methyl- BH_4 were obtained from the Schircks Laboratories (Jona, Switzerland). TNFα was measure by a Quantikine kit (R&D, MN, USA). LPS from *Escherichia coli*, sero-type 026:B6, was purchased from Sigma Chemical Company (St Louis, MO, USA). Lucigenin was obtained from Sigma (Sigma chemical company, St Louis, MO, USA) [17]. Light emission was detected with a liquid scintillation counter (Tri-Carb 2100TR, Packard, PerkinElmer, Wellesley, MA, USA). Rhodamine and dihydrorhodamine 123 were obtained from Sigma (2 μmol/kg in 0.4ml saline) (Sigma chemical company, St Louis, MO, USA), and the experiments used a multilabel counter (Model 1420; Wallac, PerkinElmer, Wellesley, MA, USA). Griess reagents were also from Sigma (1% sulfanilamide in 5% phosphoric acid, and 0.1% N-1-napthylethylenediamine dihydrochloride in water). The nitric oxide quantitation kit was from Active Motif. The kit uses a two-stop assay method that eliminates the need to use lactate dehydrogenase. This method involves the addition of two cofactors to the nitrate reductase reaction. These cofactors accelerate the conversion of nitrate to nitrite while simultaneously degrading excess NADPH to NADP.

Animals Eight-week-old female BALB/cJ mice were obtained from Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan). The mice were maintained on a 12-h light/12-h dark cycle and allowed free access to standard rodent food and water. Mice were randomly picked to different groups (PBS; LPS 10mg/kg; BH_4 10-150mg/kg with or without LPS 10mg/kg). Each group has at least 3 or more mice. Mice were given an intraperitoneal injection of LPS (10mg/kg) and different amounts of BH_4 (0, 50, 100 150mg/kg). Four hours after administration, mice were anesthetized, sacrificed and the kidneys were frozen immediately in liquid nitrogen. Blood was taken into heparinized tubes.

For bleomycin lung fibrosis model, experiments were carried out in male C57BL/6 mice weighing 25–28 g. After mice were anesthetized with ketamine and xylazine, either 50 μl of sterile isotonic saline or 0.1 U of bleomycin sulfate in 50 ml of saline per mouse was IT instilled. The mice were sacrificed by an overdose of sodium pentobarbital at 1, 3, 5, 7, 14, and 21 days after IT instillation and bronchoalveolar lavage was carried. The lung was lavaged with 1 ml of isotonic sterile saline four times. The recovery of saline ranged from 3.0 to 3.6 ml. Total cells in BALF were counted with a Coulter counter. After lavage, the lungs were dissected out, freeze clamped, and then stored at -80°C. BALF was centrifuged at 4°C for 10 min at 1500 rpm. The supernatant was gently aspirated and stored at 280°C until used

for cytokine assays [18].

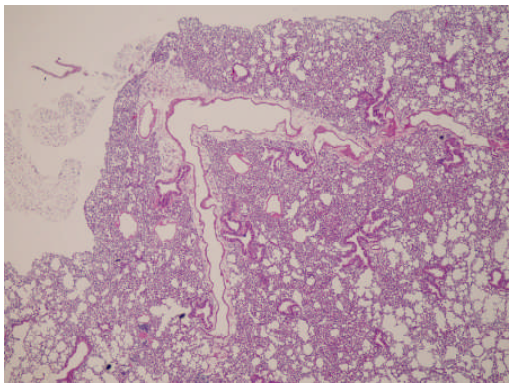
Cell cultures The mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). For experiments, cells ($1x10^6$/ml) will be resuspended with DMEM supplemented with 10% heat-inactivated FCS. Cells will be activated with LPS (1μg/ml) plus IFNγ (100 U/ml) [19].

Superoxide anion detection by chemiluminescence Chemiluminescent methods of $O_2^{\cdot -}$ detection in tissues have been used frequently because of the potential for access to intracellular sites of $O_2^{\cdot -}$ generation, the alleged specificity of reaction of the probe with $O_2^{\cdot -}$, minimal cellular toxicity, and increased sensitivity compared with chemical measurements. A thorough understanding of lucigenin-enhanced chemiluminescence requires insight into its potential reactions with $O_2^{\cdot -}$ and other molecules. The reactions involved in lucigenin-amplified chemiluminescence are as follows: reaction 1, $O_2^{\cdot -} + LC^{2+} \rightarrow LC^{\cdot +} + O_2$; reaction 2, $LC^{\cdot +} + O_2^{\cdot -} \rightarrow LCO_2$; and reaction 3, $LCO_2 \rightarrow$ 2 *N*-methylacridone + hν, where $LC^{\cdot +}$ is the lucigenin cation radical, and LCO_2 is lucigenin dioxetane. In accord with this mechanism, $O_2^{\cdot -}$ reduces lucigenin to its cation radical, which reacts with a second $O_2^{\cdot -}$ to form the energy-rich dioxetane molecule emitting a photon [17]. Plasma or scissor-minced kidney will be placed in scintillation vials containing PBS with 5μmol/L lucigenin (Sigma chemical company, St Louis, MO, USA). Light emission was detected with a liquid scintillation counter (Tri-Carb 2100TR, Packard, PerkinElmer, Wellesley, MA, USA) programmed to a single-photon-count mode. Counts were obtained at 1 minute. Background photon count was subtracted from all samples.

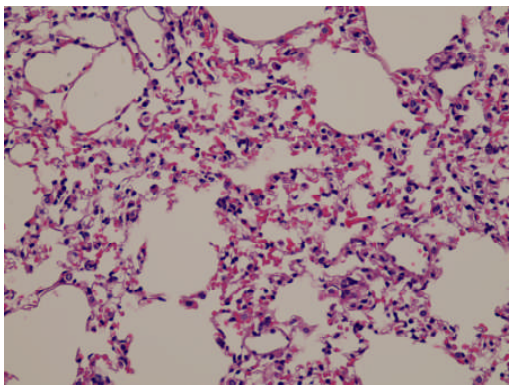
Peroxynitrite detection Dihydrorhodamine 123 (DHR) is a cell-permeant, mitochondrial-avid analog of 2,7 dichlorodihydrofluorescein that can undergo oxidation to the fluorophore rhodamine 123. The oxidized rhodamine tends to be retained within the cell after tautomerization of its equivalent amino groups. Peroxynitrite readily oxidizes DHR 123; however, several cell-derived oxidants are also capable of oxidizing DHR 123. In addition to $ONOO^-$, HOCl will also directly oxidize DHR 123. The direct reaction of DHR 123 with H_2O_2 does not occur but can be catalyzed by heme-containing peroxidases, such as HRP, or other heme compounds, including cytochrome *c*. Selective application of SOD, catalase, various NOS inhibitors, and $ONOO^-$ scavengers are required to provide for more precise identification of the substances responsible for DHR oxidation; thus, cellular

oxidation of DHR 123 to rhodamine is at best a qualitative probe for $ONOO^-$ [20]. In separate groups, mice were injected with dihydrorhodamine 123 (2 μmol/kg in 0.4ml saline, i.p.) (Sigma chemical company, St Louis, MO, USA) 4 hours after LPS and BH_4 injection. 20 minutes later, mice were sacrificed and plasma samples taken for rhodamine fluorescence evaluation using a multilabel counter (Model 1420; Wallac, PerkinElmer, Wellesley, MA, USA) at an excitation wavelength of 495nm, emission wavelength of 535nm. The rate of rhodamine formation, an index of peroxynitrite production, was calculated using a standard curve obtained with authentic rhodamine (1-30nM) (Sigma chemical company, St Louis, MO, USA) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

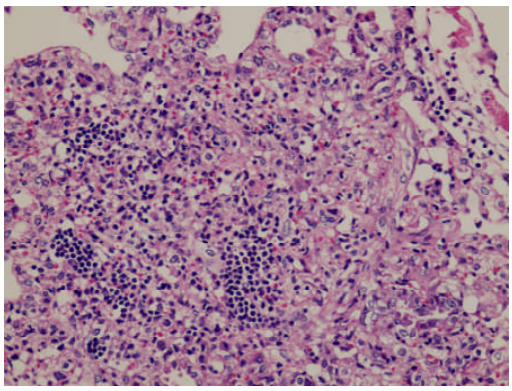
Results and Discussions



Lung fibrosis after bleomycin
B6 mice treated by 0.5U bleomycin for 2 weeks. HE stain of the lung tissues showed increase in cell infiltration and obliteration of the alveolar space

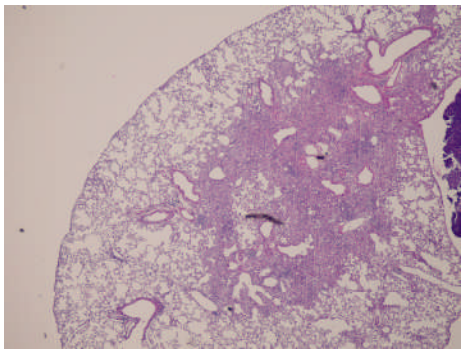


Higher magnification
The picture shows irregular alveolar sizes, increase in cell infiltration and mild hemorrhage.

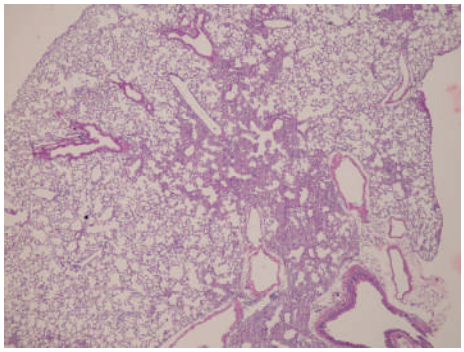


Higher magnification
An area shows complete solidification of the lung tissue.

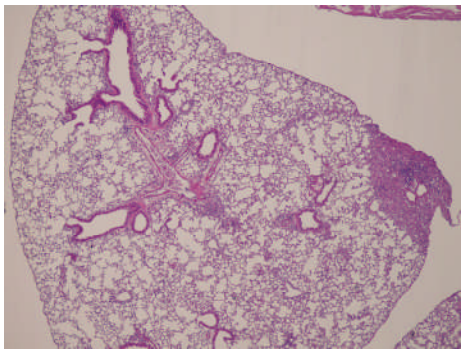
BH4 effect in bleomycin treatment



This picture shows severe localized fibrosis in the lung



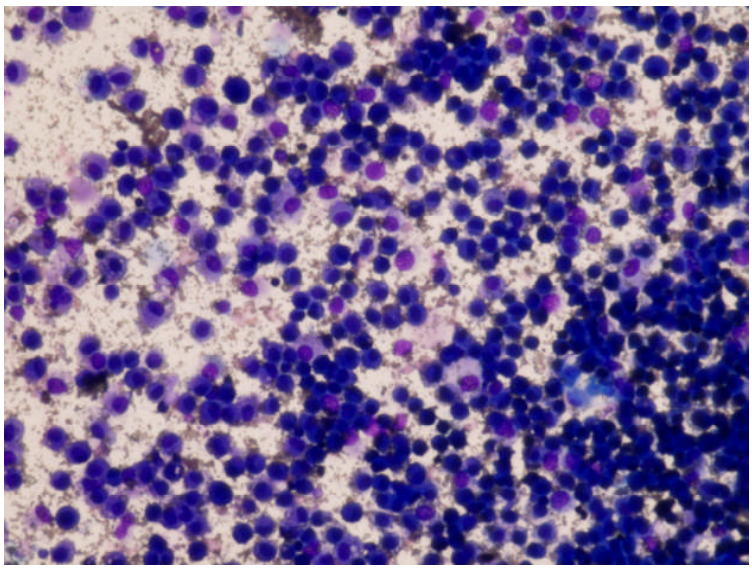
This picture shows some fibrotic areas



This picture shows one localized fibrotic area

Conclusion: BH4 increases the chance of severe focal fibrosis induced by intratracheal bleomycin treatment.

BAL



Liu stain for cells after cytospin shows mostly mononuclear cells and a small number of small lymphocytes. Neutrophil is not easily found in this picture.

BALF	Cell count	Differential count			
		Monocyte	Neutrophil	Lymphocyte	eosinphil
1.Bleomycin 0.5U-1	86	54	9	32	2
2.Bleomycin 0.5U-2	111	67	2	30	1
3.Bleomycin 0.5U-3	136	91	1	8	0
5.Bleomycin 0.5U+BH4 50mg-1	250	48	3	49	1
6.Bleomycin 0.5U+BH4 50mg-2	447	49	9	41	2
7.Bleomycin 0.5U+BH4 50mg-3	73	76	3	19	0
8.Bleomycin 0.5U+BH4 50mg-4	414	67	13	8	

Conclusion: BH4 increases the total cell count in the BAL, and also increase the percentage of small lymphocyte and neutriphil. This data indicates that BH4 increase the inflammatory response of the mice lung to bleomycin.

Discussion:

It is an alarm that BH4 increase the bleomycin-induced lung fibrosis. BH4 may increase the production of nitric oxide. BH4 itself may behave as an oxidative agent. Administration of large dose BH4 should be avoid during inflammatory status.

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