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高劑量 tetrahydrobiopterin 對發炎反應以及 nitric oxide 及自由基產生之影響

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Tetrahydrobiopterin suppresses nitric oxide production by down regulating iNOS stability

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Tetrahydrobiopterin (BH_4) is an essential cofactor for the nitric oxide synthases (NOSs). BH_4 is currently increasingly considered as a treatment module for a variety of conditions. We studied the effect of high-dose BH_4 on the production of nitric oxide (NO) and free radicals during inflammation. In mice, lipopolysaccharide (LPS) triggered the production of NO, superoxide, and peroxynitrite. Surprisingly, intra-peritoneal injection of BH_4 at a dosage of 100-150mg/kg suppressed the production of NO and the free radicals. In RAW264.7 cells treated with LPS and IFN-γ, BH_4 at a concentration of 500-750μM suppressed the production of NO, but did not alter the induction of iNOS mRNA. However, BH_4 severely shortened the half-life of iNOS protein, and dramatically decreased the steady state iNOS protein level. Therefore, BH_4 could be a potential treatment to lower down the production of NO and free radicals during acute inflammation.

The nitric oxide synthases (NOSs) are responsible for the production of nitric oxide (NO) in different tissues and cells. To date, three distinct isoforms of NOSs have been described [1]. NO produced by nNOS in neuronal tissues normally functions as a physiological mediator. NO synthesized in endothelial cells by eNOS diffuses to vascular smooth muscle where it activates the cytosolic form of guanylate cyclase to modulate vascular smooth muscle tone [2]. In macrophage, monocytes, and smooth muscle cells et al., iNOS is induced by cytokines, e.g., interferon-γ (IFN-γ) or microbial stimuli e.g., lipopolysaccharide (LPS) to produce NO which then combines with oxygen free radicals to form compounds that are more bactericidal than NO itself [3]. The expression of iNOS activity results from the induction of iNOS gene transcription [3], the inhibition of iNOS mRNA degradation [4], and/or the stabilization of iNOS protein [5].

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 [6]. The catalytic production of nitric oxide involves flavin-mediated electron transfer from C-terminal bound NADPH to the N-terminal heme center [6]. 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. Besides, BH_4 also serves as the cofactor for phenylalanine hydroxylase (PAH), tyrosine hydroxylase and tryptophen hydroxylase. Defects in BH_4 biosynthesis cause either dopa-responsive dystonia or malignant

hyperphenylalaninemia [7-10]. BH_4 has been the standard treatment for malignant hyperphenylalaninemia, and recently for BH_4-responsive type PAH deficiency [11-13].

BH_4 deficiency leads to NOS uncoupling, that is, electron is diverted to molecular oxygen rather than to L-arginine, which causes production of superoxide rather than nitric oxide [14, 15]. Superoxide reacts rapidly with NO to form the peroxynitrite anion ($ONOO^-$), which is a strong biological oxidant. Endothelial dysfunction in the insulin-resistant state has been associated with BH_4 deficiency through reduced eNOS activity and increased superoxide anion generation [16], and could be improved either by treatment with BH_4 [17], or by the overexpression of GTP-cyclohydrolase I gene [18]. More recently, it is found that statin, a drug commonly used to reduce plasma cholesterol, lowers the risk of coronary artery disease through potentiating GTP-cyclohydrolase I gene expression, BH4 synthesis, and thereby NO production [19, 20]

However, overproduction of NO is highly toxic [21]. Excessive and prolonged activation of iNOS is associated with inflammatory tissue damage in various disease states, such as septicemia, rheumatoid arthritis, and neurodegenerative diseases [22], and both inhibitors of NOS and BH4 synthesis have been used to treat these conditions [23, 24]. Therefore, BH_4 and NO would be a double-edged sword. In this study, we explored the therapeutic effect of BH_4 for acute inflammation by escalating its dosage. Interestingly, the administration of BH_4 in mice treated by LPS not only decreased the production of superoxide and peroxynitrite, but also blocked the production of NO. Similar results could be derived from RAW264.7 cells treated by LPS and IFN-γ. The suppressive effect of BH_4 was due to the down-regulation of iNOS protein stability by BH_4. The potential roles of BH_4 as a treatment for acute inflammation are discussed.

Materials and methods

Chemicals

5.6.7.8-tetrahydrobiopterin (BH_4) was obtained from the Schircks Laboratories (Jona, Switzerland). TNF-α was measured by a Quantikine kit (R&D, Minneapolis, MN, USA). LPS from *Escherichia coli*, sero-type 026:B6, was purchased from Sigma Chemical Company (St Louis, MO, USA).

Cell cultures and animal studies

The mouse macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD, USA) was maintained in Dulbecco's modified Eagle medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). For experiments, cells ($1x10^6$/ml) were treated with LPS (1μg/ml) plus IFN-γ (100 U/ml) [25].

Eight-week-old female BALB/cJ mice were assigned to different groups (phosphate-buffered saline; LPS 10mg/kg; or LPS 10mg/kg plus BH_4 10-150mg/kg). Each group constituted at least 3 mice. Four hours after intraperitoneal injection of the drugs, mice were anesthetized and sacrificed. The kidneys were frozen immediately in liquid nitrogen and blood was taken into heparinized tubes.

Nitric oxide production

Nitrite (NO_2^-) is a breakdown products of NO. Determination of plasma nitrite was performed via Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% N-1-napthylethylenediamine dihydrochloride in water) as described previously [26]. Before the assay, plasma samples were deproteined, and then reacted with the Griess reagents. The reactions were measured at A_{535} in a standard spectrophotometer.

Superoxide production

Superoxide production was measured by placing plasma or scissor-minced kidney tissues in scintillation vials containing 5μmol/L lucigenin (Sigma chemical company, St Louis, MO, USA) [27]. 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

For the measurement of peroxynitrite, mice received an intraperitoneal injection of dihydrorhodamine 123 (2μmol/kg in 0.4ml saline) (Sigma chemical company, St Louis, MO, USA) 4 hours after the treatment of LPS [28]. Twenty minutes later, mice were sacrificed and plasma samples were taken for rhodamine fluorescence evaluation using a multilabel counter (Model 1420; Wallac, PerkinElmer, Wellesley, MA, USA) at an excitation wavelength of 495nm, and emission wavelength of 535nm. Background plasma fluorescence was subtracted from all samples.

Western blot analysis and quantitative PCR

RAW264.7 cells were disrupted by ultrasound, and cleaned by centrifugation. Twenty μg of the soluble protein was subjected to western blot analysis with a monoclone anti-iNOS antibody (Cayman, Ann Arbor, MI, USA). The signals were developed using a Western Lightning kit (NEN, Boston, MA, USA)

mRNA was extracted from RAW264.7 cells with an QuickPrep Micro mRNA Purification kit (Amersham, Buckinghamshire, Egland). First strand cDNA was synthesized from the mRNA by AMV reverse transcriptase (Promega, Madison, WI, USA). Primers for the amplification of mouse iNOS and actin mRNAs were miNOS-1: 5'-CACCCTGGAGTTCACCCAGT, miNOS-2: 5'-ACCACTCGTACTTGGGATGC, mbactin-1: 5'-AGCCATGTACGTAGCCATCC, and mbactin-2: 5'-CTCTCAGCTGTGGTGGTGAA. Quantitative PCR was performed using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit and a LightCycler machine (Roche, Basel, Switzerland). Standard curves were created from purified DNA fragments for mouse actin and iNOS.

Pulse-chase study

RAW264.7 cells were seeded in 6-well plates with a density of $3x10^6$ cells per well. The cells were then stimulated with LPS and IFN-γ for 20 hours in the presence of 0, 100, or 500μM of BH_4. After treatment, the cells were incubated in serum free medium without methionine and cysteine for one hour, and then were pulsed for

45 min with S^{35}-methionine (Pro-mix, Amersham, Buckinghamshire, England). The cells were harvested immediately after pulse (time 0), or were chased in DMEM medium for 1, 2, or 6 hours. The cells were lyzed in 0.7 ml RIPA buffer (50mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, and 1 mM each of aprotinin, leupeptin, pepstatin and Na_3VO_4). Immunoprecipitation was performed with 1μl of monoclonal iNOS antibody. The final products were separated on 7.5% SDS-PAGE, and were quantified by the BAS 1500 Image Analyzer (FUJI, Tokyo, Japan).

Results

BH_4 ameliorates the LPS-triggered production of NO, superoxide, and peroxynitrite in mice

BALB/cJ mice were treated by intra-peritoneal injection of LPS (10mg/kg). Compared with saline injection, LPS triggered a 60% elevation of nitrite, three-folds elevation of superoxide, and two-folds elevation of peroxynitrite (Fig. 1). When BH_4 (10-150mg/kg) was added together with LPS in the injection, the responses to LPS were decreased in a dose-responsive manner (Fig. 1). At the highest dosage of BH_4 employed in the study (150mg/kg), the levels of nitrite, superoxide, and peroxynitrite were close to the control levels.

BH_4 abolishes the LPS/IFN-γ-triggered production of NO, superoxide, and peroxynitrite in RAW264.7 cells

RAW264.7 cells were treated with LPS 10μg/ml and IFN-γ 100U/ml for 20 hours. The amount of nitrite in the culture medium increased for more than 30 folds (Fig. 2A), and both superoxide and peroxynitrite also had a two to three folds elevation (Fig. 2B and 2C). When BH_4 50-750μM was added together with LPS and IFN-γ in the culture medium, the responses of nitrite and superoxide were suppressed in a dose-responsive manner, and BH_4 500-700μM completely abolished the production of nitrite (Fig. 2A). Peroxynitrite production was suppressed even by low-dose (50mg/kg) BH_4 (Fig. 2C).

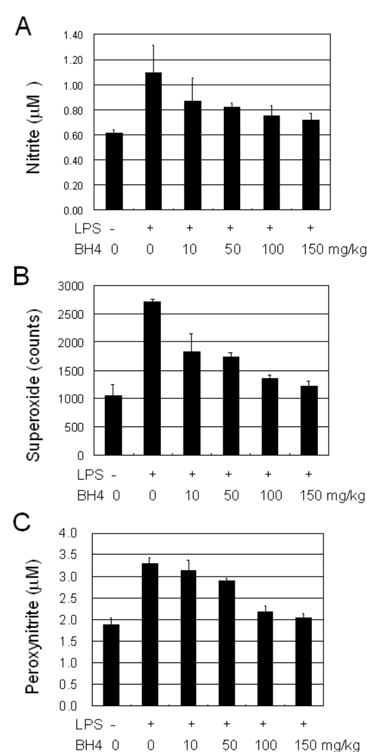

Fig. 1. Production of nitrite, superoxide, and peroxinitrite in mice. Mice were treated by 10mg/kg of LPS alone, or together with 10, 50, 75, 100, and 150 mg/kg of BH_4 for 6 hours. Both nitrite (Panels A), superoxide (Panel B), and peroxynitrite (Panel C) were measured as described in the materials and methods section.

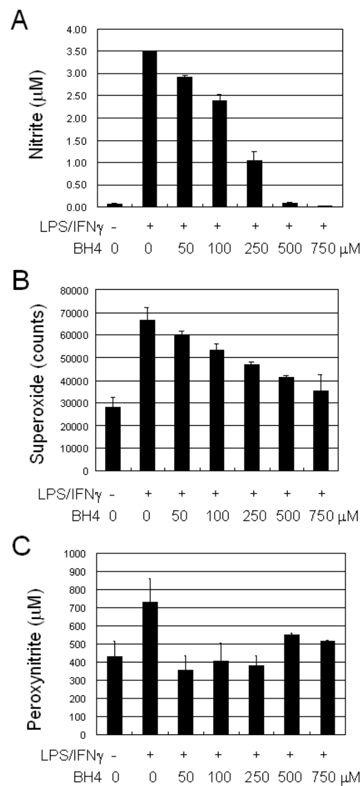

Fig. 2. Production of nitrite, superoxide, and peroxinitrite in RAW264.7 cells. The cells were stimulated by LPS plus IFN-γ for 20 hours with or without 50, 100, 250, 500 or 750 μM of BH_4. Both nitrite (Panels A), superoxide (Panel B), and peroxinitrite (Panel C) were measured at the same time as described in the materials and methods section.

BH_4 does not affect the production of TNF-α.

Large amount of TNF-α was secreted into culture medium by RAW264.7 cells after LPS/IFN-γ stimulation, as measured by an ELISA assay (Fig. 3). BH_4 treatment didn't alter the production of TNF-α (Fig. 3).

BH_4 down regulates iNOS protein stability in RAW264.7 cells.

The mechanisms for the suppression of NO production by BH_4 during inflammation were further explored by studying iNOS gene and protein expression. iNOS mRNA was not detectable by RT-PCR in resting RAW264.7 cells (Fig. 4A). After an overnight stimulation with LPS and IFN-γ, iNOS mRNA was elevated. By treatment with BH_4 during the stimulation, the amount of iNOS mRNA as measured by quantitiative PCR decreased for about 20%. This reduction cannot explain the dramatic suppression of NO production, so iNOS protein was further measured by western blot analysis. There was no visible iNOS protein in resting RAW264.7 cells, but the induction of iNOS protein by LPS and IFN-γ treatment was prominent (Fig. 4B). However, in the presence of increasing amount of BH_4, the levels of iNOS protein dropped rapidly. The results were further quantified and depicted in Fig. 4C.

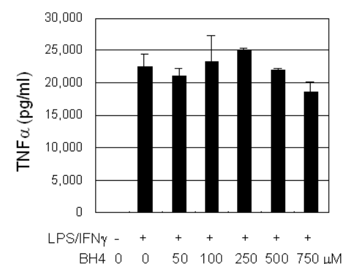

Fig. 3. Production of TNF-α by RAW264.7 cells. The cells were stimulated by LPS plus IFN-γ for 20 hours with or without 50, 100, 250, 500 or 750 μM of BH_4. TNF-α were measured in the medium by an ELISA kit.

We next pursued the etiology for the down regulation of iNOS protein by the pulse-chase study. After the LPS/IFN-γ treatment, RAW264.7 cells were pulsed with S^{35}-methionine for 45 minutes, and were either harvested immediately (pulse, time 0), or were

chased for another one, two, or six hours. The results revealed that the half-life of iNOS protein decreased dramatically from 6 hr (without BH_4) to 1.7 hr (500μM BH_4) (Fig. 4D).

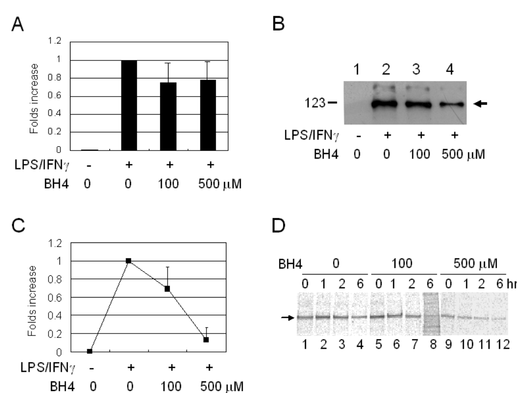

Fig. 4. Expression of the iNOS gene in RAW264.7 cells. The cells were stimulated by LPS plus IFN-γ for 20 hours with or without the presence of 100 or 500 μM BH_4. (A) Quantification of iNOS mRNA. The amounts of mRNAs were expressed relative to that of LPS/IFN-γ only. The experiments were repeated three times and the line over the bars represents one standard deviation. (B) Western blot analysis of the iNOS protein. One representative result. (C) Quantification of the results from the western blot analysis. (D) A pulse-chase study. The cells were pulsed after stimulation (time 0), and were chased for either one, two, or six hours.

Discussion

In this study in LPS inflammatory models, BH_4 treatment blocked the production of NO, but the LPS-triggered induction of iNOS mRNA and TNF-α secretion remained unchanged. In response to LPS, the cell surface Toll-like receptor 4 (TLR4) transmits the signal to cytosol, which leads to the activation of nuclear factor-κB (NF-κB) and IRF3, and then the induction of cytokines, chemokines, and other transcription factors [29]. The normal induction of iNOS mRNA and TNF-α indicated intact LPS signaling in mice and RAW264.7 cell models under BH_4 treatment. Therefore, the regulation of iNOS specifically at the post-translational level was responsible for the reduction of NO production by BH_4 treatment. Since binding of a cofactor to apoenzyme usually stabilizes the structure of the holoenzyme, the exact mechanism of iNOS destabilization certainly warrants further studies.

Upon the preparation of the manuscript, a similar suppressive effect of BH_4 on NO production was described by Thoeni et al. [30]. They used fairly low concentration of LPS (25ng/ml) without IFN-γ, and observed a decrease in iNOS and NFκB mRNA by BH_4 treatment. In our study, maximal activation of the signaling pathways by LPS/IFN-γ was used, and therefore we will have the chance to approach the destabilizing effect of BH_4 on iNOS protein.

Another important finding in this study is the suppression of superoxide production by BH_4 during inflammation. Uncoupled NOS (without adequate supply of BH_4) generates superoxide as the primary product [14, 15], but it is possible that NOS also forms superoxide under favorable conditions [31]. Therefore, BH_4 could suppress superoxide production through destabilizing iNOS. During inflammation NADPH-oxidase also generates superoxide [32], but the amount of superoxide produced by NADPH-oxidase may be not large in these LPS

inflammatory models. BH_4 is also known to undergo autoxidation and produce superoxide [33]. Therefore, it is intriguing why there is no increase in superoxide production under high dose BH_4 treatment in this study. It is possible that the autoxidation-produced superoxide was cleaned by free radical scavengers (such as superoxide dismutase) which are induced by inflammation at the same time [34].

Methods to reduce NO production include inhibition of BH_4 synthesis [23] or inhibition of NOS activity. iNOS activity can be inhibited by analogs of L-arginine [35], but the treatment induced hypertension probably by inhibiting eNOS [36]. Aminoguanidine and several other preferential iNOS inhibitors are still under investigation [37-40]. In a clinical trial in patients with septic shock, NOS inhibitor 546C88 reduced the elevated plasma nitrate concentrations and was associated with an increase in vascular tone [24]. However, the mortality rate in the experiment group paradoxically increased and the trial could not be finished [41]. In view of the failure of NOS inhibitor in the treatment of septic shock, it is very attractive to see that BH_4 could decrease NO production. BH_4 treatment may possibly provide a balance between inflammation control and NO production, and further studies approaching these possibilities should be worthwhile.

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