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Research Report

Tetrahydrobiopterin depletion and ubiquitylation of neuronal nitric oxide synthase

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Abstract

Tetrahydrobiopterin is a necessary cofactor for the synthesis of nitric oxide by the hemeprotein enzyme, NO-synthase (NOS). It is widely thought that inadequate levels of tetrahydrobiopterin lead to tissue injury and organ dysfunction due, in part, to formation of superoxide from pterin-deficient NOS. In the course of studies on the ubiquitylation of neuronal NOS (nNOS), we have found that certain substrate analogs, such as N^G-nitro-L-arginine, stabilize the dimeric form of nNOS and protect the enzyme from ubiquitylation. Since tetrahydrobiopterin is known to bind near heme and confers stability to the active dimeric structure of nNOS, we wondered if the loss of tetrahydrobiopterin could be an endogenous signal for nNOS ubiquitylation and degradation. We show here in HEK293 cells stably transfected with nNOS that depletion of tetrahydrobiopterin by treatment with 2,4-diamino-6-hydroxypyrimidine leads to destabilization of the dimeric form and enhances ubiquitylation of nNOS. Sepiapterin, a precursor to tetrahydrobiopterin in the salvage pathway, completely reverses the effect of 2,4-diamino-6-hydroxypyrimidine on nNOS ubiquitylation. Consistent with that found in cells, the in vitro ubiquitylation of nNOS by reticulocyte proteins decreases when tetrahydrobiopterin is present. Thus, inadequate amounts of tetrahydrobiopterin may lead to a sustained decrease in the steady state level of nNOS that is not readily reversed.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Other neurotransmitters

Keywords: Ubiquitin; Pterin; NO synthase

1. Introduction

Tetrahydrobiopterin is a cofactor of several amino acid metabolizing enzymes that are of importance in neurotransmitter synthesis. One of these enzymes is nitric oxide synthase (NOS), which requires tetrahydrobiopterin for metabolism of L-arginine to citrulline and NO. The importance of neuronal NOS (nNOS), inducible NOS, and endothelial NOS in neurotransmission, host defense, and vascular function, respectively, has brought much attention not only on the role of tetrahydrobiopterin deficit in a variety of diseases but also on the pharmacological supplementation of pterin (see review [32]). For example, numerous studies have described the improvement of vascular function and increased endothelial NOS activity when tetrahydrobiopterin levels are increased by pharmacological means [32]. Conversely, the depletion of tetrahydrobiopterin has also been shown to decrease endothelial NOS activity and cause endothelial dysfunction [32]. In the case of the inducible NOS, tetrahydrobiopterin depletion abrogates the ability of the enzyme to be upregulated by cytokines during an immune response [9]. The effects of tetrahydrobiopterin depletion on nNOS have not been as well characterized as the other isoforms, although an increased vulnerability to hypoxia as well as nNOS

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dysfunction is observed in neurons [5]. Overall, it is clear that an inadequate level of tetrahydrobiopterin is an important factor in a variety of pathological conditions involving NOS.

All the isoforms of NOS are ubiquitylated and proteasomally degraded [1,8,12,18]. We have recently discovered that the heme-deficient monomeric form of nNOS is preferentially targeted [2,6]. Interestingly, N^G-nitro-L-arginine, a slowly reversible, active site directed, competitive inhibitor of nNOS stabilizes the heme-containing enzyme from ubiquitylation and degradation. Thus, it appears that some conformational effect related to the heme active site confers recognition for ubiquitylation. Since tetrahydrobiopterin is known to bind near heme and confers stability to the active dimeric structure of nNOS, we wondered if changes in tetrahydrobiopterin levels could be an endogenous signal for nNOS ubiquitylation and degradation.

We have directly examined this question in a HEK293 cell line that stably expresses nNOS as well as in an in vitro degradation model containing purified nNOS and partially purified reticulocyte proteins. In the current study, we found that decreased tetrahydrobiopterin levels enhance the ubiquitylation of nNOS in both models. Furthermore, tetrahydrobiopterin depletion in cells also leads to destabilization of the dimeric form of nNOS, but not to the loss of the prosthetic heme from nNOS. This indicates that a pool of inactive, heme-containing nNOS, which is not in a tightly associated homodimeric form, exists in cells. It appears that this pool of destabilized nNOS is labilized or susceptible to ubiquitylation and proteasomal degradation. Thus, the loss of nNOS protein should be considered as a long-term consequence of inadequate tetrahydrobiopterin levels in a variety of pathological and toxicological conditions.

2. Materials and methods

2.1. Materials

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2,4-diamino-6-hydroxypyrimidine, calmodulin (crude, from bovine brain), horse heart myoglobin, anti-βtubulin antibody, L-arginine, leupeptin, NP40 (IGEPAL CA-630), A23187, ATP, ubiquitin, $MgCl_2$, creatine phosphokinase, hexokinase, and NADP+ were purchased from Sigma. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin and sepiapterin were purchased from Dr. Schirck's Laboratory (Jona, Switzerland). The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from Transduction Laboratories (Lexington, KY). ^{125}I-labelled goat antibody against rabbit IgG or mouse IgG was purchased from Perkin Elmer (Boston, MA). The affinity-purified rabbit IgG used for Western blotting of ubiquitin was from DAKO Corporation (Carpinteria, CA). The rabbit antiserum used to immunoprecipitate nNOS was raised against rat neuronal NOS and was the generous gift of Dr. Lance Pohl (NHLBI, Bethesda). The antibody was affinity-purified prior to use. Peroxidase-conjugated anti-rabbit IgG antibody was from Boehringer Mannheim (Indianapolis, IN). Ubiquitin aldehyde was from Alexis Biochemicals (San Diego, CA). Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). DE52 was purchased from Whatman Inc. (Fairfield, NJ). Cbz-leucine-leucine-leucinal (MG132) was purchased from BIOMOL (Plymouth Meeting, PA).

2.2. Cell culture and preparation of the cytosolic fraction

Human embryonic kidney 293 cells (HEK293) stably transfected with rat nNOS by Bredt et al. [3] were obtained from Dr. Bettie Sue Masters (University of Texas Health Science Center, San Antonio, TX). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone®), 20 mM HEPES, pH 7.4, and G418 (0.5 mg/ml, Geneticin®, Life Technologies, Inc.) as described previously [17]. Prior to each experiment, the cells were cultured in DMEM containing 0.1 mM L-arginine (low arginine DMEM) for at least 12 h. HEK cells were treated with 5.0 mM 2,4-diamino-6-hydroxypyrimidine (DP) and 100 μM sepiapterin (SP) similar to that previously used for other cells [9,25]. There was greater than 85% cell viability, as determined by trypan blue, for all conditions used in our studies. Cell viability was unaffected by DP or SP treatment. Sepiapterin was added in DMSO and the total concentration of DMSO did not exceed 0.2% in the medium. DMSO alone did not have any effects on the amount of monomeric or dimeric nNOS. HEK cells were harvested in their treatment medium, diluted 1:1 with ice-cold phosphate-buffered saline. The cells were then pelleted, washed 3-times with 5 ml of ice-cold phosphate-buffered saline, and pelleted again. The cell pellet was homogenized on ice with a Tenbroeck ground glass homogenizer in three volumes of lysis buffer containing 50 mM Tris–HCl, pH 7.4, 1.0 mM EDTA, 1.0 mM DTT, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged for 20 min at 16,000 × g and the supernatant was used for assays. For HPLC studies, the supernatant was removed and centrifuged for an additional 15 min at 100,000 × g to obtain a cytosolic fraction. For ubiquitin studies, the cell pellet was homogenized in HE lysis buffer containing 10 mM HEPES, pH 7.4, 0.32 M sucrose, 2.0 mM EDTA, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM *N*-ethylmaleimide (NEM), 10 mM Na_3VO_4, 1% NP40, and 6 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged for 20 min at 16,000 × g, the supernatant was used for assays.

2.3. Assay for SDS-resistant dimer of nNOS

To detect the SDS-resistant dimer of nNOS, we used an SDS-PAGE method previously described by Klatt et al.