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The role of tryptophan metabolism in iNOS transcription and nitric oxide production by chicken macrophage cells upon treatment with interferon gamma

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Abstract

The influence of *de novo* synthesis of nicotinamide adenine dinucleotide (NAD) through the kynurenine (KYN) pathway of tryptophan (TRP) degradation on gene transcription of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production in chicken interferon gamma (ChIFN-γ)-stimulated and non-stimulated chicken macrophage cell line HD11 was investigated. Interferon gamma up regulation of iNOS transcription and NO production was dependent on an undisturbed flow through the KYN pathway. Inhibition of indoleamine-2,3-dioxygenase, the rate-limiting enzyme of TRP catabolism, by 1-methyl-L-tryptophan (1-mTRP) down regulated both iNOS gene transcription and NO production. Addition of KYN to 1-mTRP-treated, ChIFN-γ-stimulated macrophages circumvented the down regulation of iNOS transcription and NO production. Inhibition of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, replication and transcription, which cleaves NAD into nicotinamide and ADP-ribose, down regulated iNOS gene transcription and NO production in ChIFN-γ-stimulated HD11 cells. Our results suggest that prevention of NAD depletion in HD11 cells by ChIFN-γ-mediated induction of IDO facilitates iNOS transcription and NO production. This effect is most likely a result of PARP1 automodification in the presence of NAD, known to facilitate transcription by changing chromatin structure and to allow NFκB binding to iNOS promoter which is hindered by direct protein–protein interaction between NFκB and unmodified PARP1.

Keywords: Tryptophan metabolism; Poly(ADP-ribose) polymerase; Inducible nitric oxide synthase; Chicken macrophage

1. Introduction

Upon immune stimulation, interferon gamma (IFN-γ) up regulates transcription of a number of genes, among which are those for indoleamine-2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS) [1,2]. Indoleamine-2,3-dioxygenase is the rate-limiting enzyme in the kynurenine (KYN) pathway of tryptophan (TRP) degradation to nicotinamine adenine dinucleotide (NAD) [3]. The role of IDO in immune responses has been attributed to TRP depletion and/or production of immunosuppressive metabolites [4–6]. Nitric oxide (NO), a signaling molecule involved in numerous physiological and pathophysiological processes, is produced from L-arginine (L-ARG) by NOS [7]. Inducible NOS is activated in macrophages upon immune stimulation to produce large amount of NO, one of the main mediators of inflammatory responses [8]. The KYN pathway of L-TRP degradation to NAD and L-ARG degradation to produce NO and are tightly interconnected in maintenance of cell homeostasis. Tryptophan degradation leads to *de novo* synthesis of NAD(P), one of the cofactors needed for iNOS activity [9], while the NO produced by iNOS inhibits IDO by interacting with superoxide [10]. Nitric oxide and superoxide, produced upon stimulation with IFN-γ, can cause self-inflicted oxidative damage, DNA strand breaks and an increase in poly(ADP-ribose) polymerase (PARP) activity, leading to NAD depletion. It has been proposed that enhanced synthesis of NAD by macrophages during an immune response is a self-protecting mechanism [11]. Activated PARP-1 hydrolyzes NAD and transfers the ADP-ribose moieties to form poly(ADP-ribose) on acceptor proteins such as histones, DNA repair proteins, transcription

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factors and PARP-1 itself [11–14]. The concentration of NAD has been shown to influence the length of poly(ADP-ribose) synthesized by PARP-1 *in vitro* [15], as well as to regulate chromatin structure and transcription [16]. The automodification of PARP-1 is known to prevent its direct protein–protein interaction with transcription factor NFκB that renders it incapable of binding to DNA and executing its transcriptional activity [17], hence poly(ADP-ribosyl)ation positively regulates iNOS gene transcription. Poly(ADP-ribose) polymerase executes its transcription-regulatory role also by down regulating catalytic activity of DNA methyltransferase 1 [18]. Since, hypermethylation of the iNOS gene promoter inhibits iNOS gene transcription [19], poly(ADP-ribose)-mediated up regulation of iNOS transcription may be yet another link in immune regulatory relationship of L-ARG and L-TRP metabolic pathways.

2. Materials and methods

2.1. Cells and culture conditions

The replication-defective avian leukemia virus MC29-transformed macrophage cell line, HD11 (a gift from Dr. Kaspers, University of Munich, Germany) was used in this study. MC29-transformed cells are somewhat less mature than fully differentiated macrophages. The difference between MC29-transformed macrophage-like cells and their normal counterparts is that they are less adherent, divide more rapidly and that the former can spontaneously differentiate to the latter. They express Fc receptors, have phagocytic capacity and have macrophage cell surface antigen, while expressing only weakly myeloblast cell surface antigen and are negative for ATPase activity [20]. Cells were maintained in RPMI 1640 medium, containing L-glutamine, sodium bicarbonate, 8% fetal bovine serum, 2% chicken serum and antibiotics, were kept at 41 °C (optimal temperature for avian cells) in a water-saturated 5% CO_2/95% air atmosphere, with regular passage.

2.2. Cell stimulation with IFN-γ and inhibition of PARP

Cells were grown to confluence, culture medium containing non-adherent cells was removed, and the cells were detached from culture flasks by incubating with phosphate-buffered saline (PBS)—0.5 mM EDTA for 5 min at 37 °C. PBS-EDTA was removed by centrifugation at 1200 rpm for 5 min, 4 °C, cells were washed with fresh medium, counted, and 2 ml of 10^6 cells/ml were placed into each well of 24-well plate. Cells were grown in the presence of various combinations of TRP, 1-mTRP and KYN, added separately to the medium. Finally, cells were stimulated with 100 ng/ml of recombinant ChIFN-γ. In some experiments, a PARP inhibitor, 1,5-isoquinolinediol (ISO), 100 μM, was added to culture media 2 h prior to stimulation with IFN-γ.

2.3. Measurement of NO synthesis and release

The levels of NO that accumulated in cell culture media were determined by the Griess reaction adapted to microtiter plate format [21]. Briefly, 100 μl of conditioned medium were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N (1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid) at room temperature for 10 min. Absorbance was measured at 570 nm, using a microtitre plate reader. Obtained absorbances were converted to micro molar values using the slope of a calibration curve established by serial dilutions of sodium nitrite from 250 to 1.95 μM.

2.4. Measurement of IDO activity

The activity of IDO was measured by the method of Takikawa et al. [3] as modified by Kudo and Boyd [22]. Briefly, cells were washed twice and resuspended in 1 ml of PBS. The cells were disrupted by sonification for 30 s on ice at 100 W. The homogenate was centrifuged at 12,000 × *g* for 5 min at 4 °C. Protein content of the supernatants was determined using Bradford reagent and bovine serum albumin as standard. Supernatants and incubation buffer containing 1 mM L-tryptophan, 20 μM methylene blue, 40 mM ascorbic acid, catalase (200 U/ml) and 100 mM potassium phosphate buffer, pH 6.5, were pre-heated to 37 °C before mixing 0.4 ml of each sample with an equal volume of incubation buffer. The reaction was carried out for 30 min at 37 °C and stopped by adding 0.2 ml of 30% (w/v) trichloroacetic acid. The mixture was incubated for 30 min at 50 °C to hydrolyze *N*-formylkynurenine produced by IDO to kynurenine. The mixture was centrifuged at 12,000 × *g* at room temperature, and 0.8 ml supernatant was combined with 0.8 ml freshly prepared 1% (w/v) *p*-dimethylaminobenzaldehyde in glacial acetic acid. Absorbance at 492 nm was measured using a microtiter plate reader.

2.5. Measurement of KYN in culture media

The KYN concentration in the culture media was assayed spectrophotometrically [23]. Briefly, 200 μl of culture supernatant were mixed with 100 μl of 30% trichloroacetic acid, and centrifuged at 10,000 rpm for 5 min. Supernatant (125 μl) was mixed with an equal volume of Erlich's reagent (1% (w/v) *p*-dimethylaminobenzaldehyde in glacial acetic acid) and absorbance at 492 nm was measured against a reagent blank in a microtiter plate reader. The concentration of KYN in the samples was estimated using calibration curve established by serial dilutions of KYN from 10 to 0.01 μM.

2.6. Measurement of NAD

Pyridine nucleotide content (NAD and NADH) was estimated by the thiazolyl blue micro cycling assay [24] modified to a microtiter plate format. Briefly, 10^6 cells were homogenized in 1 ml PBS containing PARP inhibitor by sonication for 30 s on ice at 100 W. The reaction mixture (125 μl), composed of ethanol (120 μM/ml), alcohol dehydrogenase (1 mg/ml), phenazine methosulfate (1 mg/ml), and MTT (0.2 mg/ml) in bicine-buffered solution (pH 7.8), was added to 20 μl of cell homogenate. Alcohol dehydrogenase converted available NAD+ in the sample to the reduced form (NADH). Formazan