Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2001;9:203–208

Received: February 12, 2001
Accepted: September 11, 2001

Influence of Reduced Nicotinamide Adenine Dinucleotide on the Production of Interleukin-6 by Peripheral Human Blood Leukocytes

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Key Words

Inflammation · Interleukin-6 · Neurodegenerative disorders · Reduced nicotinamide adenine dinucleotide · NADH

Abstract

Objective: Recently, therapy with nicotinamide adenine dinucleotide (NADH) revealed positive effects on neurodegenerative disorders associated with inflammation of the CNS, such as Parkinson's disease or Alzheimer's disease. Pathophysiologically, focal CNS inflammation seems to be accompanied by an unbalanced cytokine production, pointing to an involvement of the immune system. Therefore, the aim of our study was to investigate whether NADH could influence cytokine release of peripheral blood leukocytes (PBLs) with special reference to interleukin-6 (IL-6). ***Methods:*** PBLs from 18 healthy donors were incubated in vitro with different concentrations of NADH to generate dose-response curves. As a control, mitogen-treated cells and unstimulated cells were included. ***Results:*** In PBLs from the 18 healthy donors, NADH significantly stimulated the dose-dependent release of IL-6, ranging from 6.25 to 400 μg/ml, compared to medium-treated cells ($p < 0.001$). An amount of 1,000 pg/ml IL-6 was induced by NADH concentrations ranging from 3.1 to >25 μg/ml. ***Conclusions:*** It is concluded that NADH possesses cytokine-modulating effects on peripheral blood cells. The biological relevance of these data is discussed in the context of the recent use of NADH for the treatment of several neurodegenerative disorders.

Introduction

Among the numerous enzymes participating in energy production of living cells, the redox system nicotinamide adenine dinucleotide (NAD^+/NADH) plays a fundamental role for oxidative phosphorylation [1] leading to the generation of adenosine triphosphate (ATP). Recently, NADH (synonyms β-NADH, reduced DPN) has been described to stimulate the biosynthesis of neurotransmitters [2]. They are essential to maintain normal neuronal functions, thereby ensuring the individual's capabilities to preserve locomotion, and mental and cognitive behavior. Accordingly, NADH has been used previously as a neurotherapeutic approach to several diseases of the central nervous system [3–5] associated with an altered dopamine, serotonin and norepinephrine metabolism [6, 7]. In Parkinson's disease patients, who are characterized by a deficit in dopaminergic neurons in the substantia nigra of the brain [8], an improvement in patients' symptoms following NADH treatment was found to be related to an enhanced endogenous dopamine production, reflected by

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1021–7401/01/0094–0203$17.50/0

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an increase in the urinary excretion of dopamine metabolites, such as homovanillinic acid [3, 8]. These findings suggested a positive effect of NADH on these CNS disorders. In addition, some publications favor an additional role of the immune system in disease progression in the brain [9]. However, the role of distinct cytokine proteins in the induction or maintenance of brain inflammation and homeostasis is currently disputed [10, 11]. Particularly for interleukin-6 (IL-6) multiple functions in the immune, hematopoietic and nervous system have been shown, e.g. its neurotrophic activities on dopaminergic neurons [12]. The question of whether NADH, beside its recently found effects on the endogenous dopamine metabolism, could also affect immunoregulatory cytokines arises. This is the more interesting as some newly published reports demonstrated that neurodegenerative disorders may also have a systemic expression, which is shown by phenotypic/functional changes in leukocytes with regard to phagocytosis, apoptosis and cytokine production, compared to healthy individuals [13–15]. Therefore, peripheral blood leukocytes of healthy donors were used as target cells to examine the effects of NADH on IL-6 production. The aim of the study was to examine whether NADH could affect the release of IL-6 by peripheral blood leukocytes (PBLs).

Materials and Methods

Test Substances

For the in vitro experiments NADH (as disodium salt) was purchased from Biomol and used at a final concentration of 400 μg/ml in the first screening experiments to evaluate which cytokine, if any, might be affected by NADH. In the second series of experiments, NADH was serially 4-fold diluted with complete cell culture medium (400 to 0.024 μg/ml). The positive control was a mixture of the following lectins, each diluted to suboptimal concentrations: phytohemagglutinin, concanavalin A and pokeweed mitogen, all purchased from Seromed, Berlin (Germany), were used at 200 ng/ml, respectively. Lipopolysaccharide (LPS, *Escherichia coli* serotype 55:B5, Sigma/Aldrich Chemicals, München, Germany) was used at a final concentration of 200 ng/ml.

Preparation of PBLs

PBLs from 4 (first screening experiment) and 18 (second experiment) healthy human donors, supplied as buffy coats, were isolated by density centrifugation of heparinized blood over Ficoll-Hypaque (Pharmacia, Freiburg, Germany) using 30 ml blood cells each diluted 1:2 with phosphate-buffered saline (PBS). After centrifugation (20 min, 350 *g*, 20°C), leukocytes of the interphase were recovered, resuspended in PBS and washed three times with PBS. Thereafter, the cell number was adjusted to 2×10^6 cells/ml with cell culture medium, consisting of RPMI-1640, supplemented with 5% heat-inactivated (1 h, 56°C) fetal calf serum, 100 μg/ml gentamycin, 1 m*M* natrium pyruvate and 2 m*M* N-acetyl-*L*-alanyl-*L*-glutamine (all purchased from Seromed). Leukocytes (2×10^6/ml, final volume 3 ml) were cultivated in 24-well culture plates (Nunc, Wiesbaden, Germany) at 37°C, 5% CO_2 in a humidified atmosphere over a period of 72 h in the first experiment and for 24 h in the second experiment.

Treatment of Human Leukocytes with the Test Substances

During the first screening, six different cytokines were examined: tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-10, IL-2 and interferon-γ (IFN-γ). In these experiments, only one concentration of NADH (400 μg/ml) was used. NADH was tested either with, or without the addition of the positive control to assess the effects of NADH on polyclonally stimulated cells, too. Non-stimulated cells were used to detect the background production of cytokines. In the second part of the study, the influence of NADH particularly on IL-6 production of PBLs (2×10^6 cells/ml, final volume 3 ml) was examined. The main purpose of these experiments was to establish dose-response curves for NADH with PBLs of individual donors. Hence, PBLs were stimulated with increasing concentrations of NADH, from 0.024 to 400 μg/ml to determine the NADH concentration able to stimulate the release of 1,000 pg/ml IL-6. Presently, NADH was tested only on unstimulated cells (n = 3), and parallel cultures from cells of each donor received the positive control, e.g. the mixture of lectins (n = 3) to assess the maximal release of IL-6 under these experimental conditions. Controls received medium (n = 6). According to previous studies with cytokine release under these conditions, TNF-α was determined after 6 h, IL-1β, IL-2 and IL-6 were measured after 24 h of incubation, IL-10 after 48 h of incubation and IFN-γ after 72 h of incubation. In the second experiment, IL-6 was determined after 24 h of incubation. Cell culture supernatants were harvested by centrifugation, (1,200 rpm, 10 min, 4°C) and aliquots were stored at −80°C until determination of all cytokines with specific sandwich ELISA tests. The viability of the cells was routinely tested with all cell cultures to exclude toxic effects of NADH, leading to incorrect interpretation of the data with respect to cytokine release. The viability tests, using propidium iodide staining with subsequent FACS-analysis, revealed a percentage of viable cells >85% in the stimulated cultures and >90% in non-stimulated cell cultures.

ELISA Procedures

We chose to determine the cytokine production at the protein level, where the secreted protein can be measured by ELISA. The ELISA tests were all supplied by Biosource/Medgenix (Nivelles, Belgium). The sensitivity ranges of each ELISA test were between 8,000 and 125 pg/ml for TNF-α, IL-6 and IL-10, respectively. For IL-1β, IL-2 and IFN-γ the sensitivity ranged between 4,000 and 62.5 pg/ml. All ELISA assays were strictly performed according to the recommendations of the manufacturer. Briefly, coating of the ELISA plates (Maxisorb, 96-well plates, Nunc), was done at pH 9.6 in PBS/carbonate buffer (Sigma/Aldrich Chemicals, München, Germany) for 2 h at room temperature (RT) with the respective first antibody (mouse anti-human TNF-α, mouse anti-human IL-1β, mouse anti-human IFN-γ, rat anti-human IL-2, rat anti-human IL-6 and rat anti-human IL-10, all supplied by PharMingen, Hamburg, Germany). Coating of plates was performed with 1 μg/ml antibody (50 μl/well) for the determination of IL-1β, TNF-α, IL-6, IL-10 and IFN-γ. For the IL-2 ELISA, 2 μg/ml antibody (50 μl/well) was used. After blocking of non-specific binding sites with 1% (w/v) natrium-casein solution (Sigma/Aldrich Chemicals) for 60 min (100 μl/well) at 37°C, plates

were washed twice with 0.2 ml 0.05% (v/v) PBS/Tween 20 (PBST, Sigma/Aldrich Chemicals), followed by the addition of the standards: human rIL-1β (Boehringer Mannheim, Mannheim, Germany), human rTNF-α, human rIL-2, human rIL-6, human rIL-10 and human rIFN-γ (all purchased from PharMingen), or culture supernatants, appropriately diluted in RPMI-1640 (50 µl/well, triplicate determinations). After 2 h of incubation at RT and three washes with 200 µl/well PBST, the biotin-labeled second antibodies (50 µl/well) were added, each diluted with 0.05% PBST/ 0.1% (v/v) natrium-caseinate solution: goat anti-human IL-1β (50 ng/ml, R & D Systems, Wiesbaden, Germany), rabbit anti-human IL-2 (1 µg/ml), rat anti-human IL-6 (125 ng/ml), rat anti-human IL-10 (400 ng/ml), murine anti-human IFN-γ (1 µg/ml) and murine anti-human TNF-α (100 ng/ml), all purchased from PharMingen. Then, the plates were incubated for 2 h on a microplate shaker (300–400 rpm) followed by three washing steps each with PBST (0.05%, v/v). The enzyme solution (streptavidine/peroxidase, Jackson Immuno Research, USA, 250 ng/ml, 100 µl) prepared in 0.05% PBST/0.1% natrium-caseinate was added for 1 h at RT, followed by four washes with 0.2 ml PBST (0.05%, v/v). Finally, the substrate solution (tetramethylbenzidine, Fluka via Sigma/Aldrich Chemicals) was added (100 µl/well) and the plates were incubated at RT in the dark. The reaction was stopped with 50 µl 2 *N* H_2SO_4 (Merck, Darmstadt, Germany). The optical density was measured at 450 nm in a microplate reader (Titertek Multiscan®, Dynatech, Germany).

Statistical Analysis

The dose-dependent relationships between NADH-induced IL-6 production and intersubject differences were ascertained by means of statistical calculation programs as LIMDEP software. Repeated measure analyses of variance (ANOVA) were used to analyze variations between individual measurements and to estimate between-subject effects. In a post-hoc analysis, homogeneity between subjects in response to the different amounts of NADH were analyzed with Student-Newman-Keuls and Tukey HSD. Results were expressed as means ± SD. $p \leq 0.05$ was considered as statistically significant.

Results

Influence of NADH on the Release of the Cytokines IL-1β, TNF-α, IL-2, IL-6, IL-10 and IFN-γ

The first experiment with NADH revealed considerable differences with some interindividual variations concerning the stimulating effects of NADH on cytokine production of PBLs from the 4 donors (data not shown). The cytokines IL-1β and IL-10 were stimulated more or less by NADH alone with no additional increase after costimulation with the mixture of lectins. The synthesis of IL-2 remained unchanged after addition of NADH as well as under conditions of costimulation, and TNF-α and IFN-γ seemed to be diminished by about 10–30% only in mitogen-stimulated cells from some donors. The most remarkable finding, which was consistently found in leukocytes from all 4 donors, was the pronounced enhancement of IL-6 production by PBLs treated with NADH alone. Hence, in the second series of experiments, only the IL-6 production of PBLs stimulated by NADH alone was examined.

Influence of Different Concentrations of NADH on the IL-6 Production of Human PBLs from 17 Separate Donors

The second series of experiments confirmed and extended the preliminary results from the first observations in that NADH promoted dose-dependently the release of IL-6 in PBL cultures from all 17 donors. PBLs from 1 donor were omitted from further calculations of the dose-response curves due to an unacceptable background stimulation in IL-6 release. Figure 1 exemplifies dose-response curves with PBLs from 11 individual donors demonstrating that the response of leukocytes to the lower concentrations of NADH varied considerably between the individual donors. Analysis of this effect of NADH by means of ANOVA and the method of repeated measurements revealed significant differences in IL-6 production between PBLs of individual donors, ranging from 25 to 400 µg/ml NADH ($p < 0.05$). Further analysis of between-subject effects however showed clearly that NADH at concentrations ranging from 6.25 to 400 µg/ml was able to significantly stimulate the release of IL-6 above background values ($p < 0.001$), suggesting that a concentration of 6.25 µg/ml NADH seems to be a critical cutoff value, as IL-6 production was negligible with PBLs from most of the donors below that NADH concentration. In table 1, the concentrations of IL-6 (means ± SE, triplicate determinations) were summarized using 400 µg/ml NADH to illustrate the potency of NADH. The data were expressed with respect to the positive control, which was ascribed 100%.

PBLs from 3 donors (2, 6 and 17) released IL-6 concentrations in the range of the positive control indicating a high activating potential of NADH, whereas the lowest induction of IL-6 synthesis upon stimulation with NADH was observed with PBLs from donors 8, 10, 12, 14 and 15 (maximally a third of the positive control). However, the great majority of PBLs produced about half of the amount of IL-6 which could be maximally induced by polyclonal stimulation. No toxic effects were observed in the cell cultures after staining for viability and FACS analysis (data not shown), even with 400 µg/ml NADH. Therefore, the aim of the second part of the experiments was to determine the NADH concentration which is able to stimulate a biologically relevant IL-6 production, which was chosen as 1,000 pg/ml according to plasma levels of IL-6 cited in the literature. Based on linear regression analysis of the 17

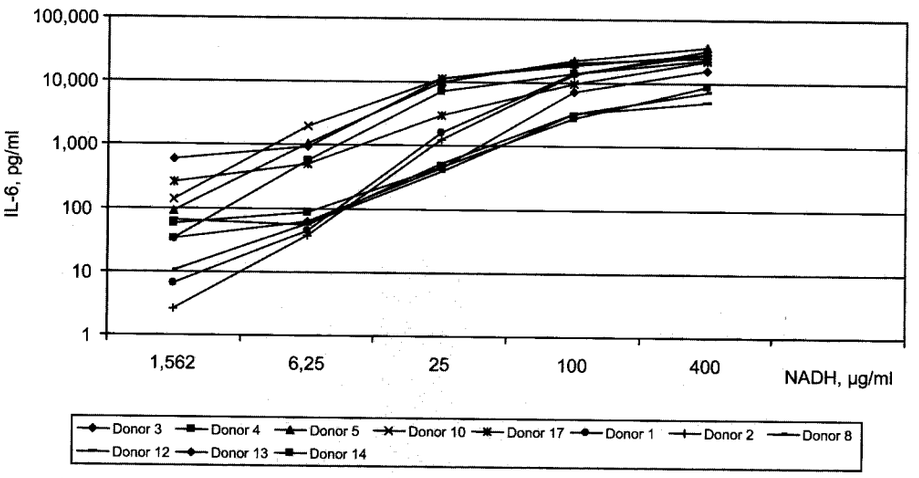

Fig. 1. Dose-response curves obtained with increasing concentrations of NADH (µg/ml) using PBLs from 11 individual healthy donors. Concentrations of IL-6 (pg/ml) were determined in triplicate. The dose-response curves of the remaining donors corresponded to those shown and were therefore omitted. Please note that IL-6 concentrations were shown in a logarithmic scale.

Table 1. Maximal release of IL-6 (pg/ml) by human PBLs stimulated with 400 µg/ml NADH compared to IL-6 production induced by the positive control and estimated amount of NADH able to stimulate 1,000 pg/ml IL-6

Donor No.	IL-6, pg/ml, after stimulation with 400 µg/ml NADH[a]	Background stimulation[b]	Positive control[c]	Percent of positive control	Estimated NADH concentraton able to induce 1,000 pg/ml IL-6[d]
1	28,481.6±3,553.7	0±0	38,212.3±495.2	74.5	12.5–25
2	32,275.5±14,590.9	0±0	19,673.0±755.0	164.0	12.5–25
3	28,893.8±2,180.4	0±0	54,337.9±7,749.1	53.1	3.1–6.2
4	23,240.6±2,754.5	7.7±11.6	34,484.2±3,145.4	67.3	3.1–6.2
5	36,307.6±2,114.4	0±0	44,030.9±7,123.4	82.4	3.1–6.2
6	28,608.9±1,382.9	19.0±26.2	26,184.3±5,073.8	109.2	6.2–12.5
7	16,094.0±1,040.8	0±0	32,178.4±3,809.2	50.0	12.5–25
8	7,206.3±560.7	0±0	24,279.0±1,031.3	29.6	>25
9	25,392.9±4,205.1	0±0	31,284.0±2,234.1	81.1	6.2–12.5
10	25,305.2±2,217.8	44.1±21.2	73,082.9±8,505.7	34.6	3.1–6.2
11	11,326.5±170.1	13.5±6.5	22,362.6±4,131.8	50.6	12.5–25
12	4,963.4±2,273.6	81.5±30.9	35,282.8±1,864.0	14.0	>25
13	15,874.3±713.9	61.3±17.3	39,446.6±4,128.1	40.2	>25
14	8,749.1±258.1	24.8±7.1	31,342.8±806.8	27.9	>25
15	12,257.6±1,350.3	23.7±8.5	35,277.6±7,407.6	34.7	12.5–25
16	11,099.6±665.1	10.3±6.4	23,369.3±975.8	47.4	12.5–25
17	21,955.4±4,956.5	61.3±35.4	19,673.0±755.0	111.6	3.1–6.2

[a] Mean values of triplicate determinations and standard error of the mean.
[b] Mean values of six determinations and standard error of the mean.
[c] Mean values of triplicate determinations and standard error of the mean.
[d] Estimated concentration of NADH according to the regression curves calculated for each donor.

dose-response curves, concentrations of NADH stimulating 1,000 pg/ml IL-6 were estimated for each donor (table 1). They varied between 3.1 μg/ml and >25 μg/ml NADH.

Discussion

The results of this study demonstrate that NADH was able to stimulate dose-dependently the production of IL-6 by human peripheral blood leukocytes. Although the exact mechanism(s) of NADH-induced IL-6 stimulation is unknown at present, NADH may possibly increase IL-6 synthesis and release by PBLs at the level of transcription. Since the molecular cloning of IL-6 in 1986 [16], knowledge on the signal transduction pathways initiated after binding of IL-6 to its receptor has increased considerably [17]. Due to this increased knowledge on signal transduction pathways combined with the fact that NADH is ultimately involved in the mitochondrial generation of ATP it may be hypothesized that ATP activates distinct ATP-sensitive transcription factors possibly increasing the synthesis of IL-6. Support for this mere speculation may be derived from findings recently published by Tanaka et al. [18] demonstrating that survival of striatal neurons after focal cerebral ischemia in the rat was specifically mediated by a family of transcription factors activated by a cyclic AMP-response element binding protein. In the past, the research on possible neuroprotective roles of this extremely pleiotropic cytokine has received considerable attention from neuroscientists and resulted in a lot of publications with contradictory results, underlining the complex nature of the communication between the CNS and the immune system. A lot of experimental studies in vitro [19–21] and animal studies [22–24] indicated a potential neuroprotective role of IL-6 in several forms of neuronal injury. In contrast, in clinical situations of neurodegenerative disorders including Alzheimer's disease, stroke or Parkinson's disease, the role of IL-6 and its soluble receptor becomes the more puzzling as IL-6 was either reported to be diminished or increased [25–28]. Thus, although IL-6 protein and gene expression together with its soluble receptor have been identified in several CNS regions, where both proteins seem to act as mediators of host defense and affect neuronal survival, the putative protective role of IL-6 remains not fully understood [29, 30]. However, the limitations of using peripheral leukocytes and not brain-derived target cells, such as glia cells, neurons or brain-derived endothelial cells, should be borne in mind. Moreover, the question of whether NADH-induced peripheral IL-6 may be able to affect neurodegenerative disorders in the CNS remains to be determined. However, a prerequisite for the assumed protective role of systemically induced IL-6 to counteract local inflammation in the brain may be either its passage across the blood-brain barrier and/or a migration of IL-6-producing cell types into the brain parenchyma, a hypothesis which has been supported by several recent publications [31–33]. It may also be conceivable that the normally intact blood-brain barrier may be entered by cytokines and activated leukocytes under conditions of peripheral or local inflammation [34]. Accordingly, severe traumatic brain injuries could disrupt the normally intact blood-brain barrier [35]. Notably, early IL-6 production in the CNS was found by this study group [35] to exceed serum IL-6 levels by several orders of magnitude. A correlation between CSF and serum IL-6 was found by Kossman et al. [35] only initially after trauma and corresponded to a severe dysfunction of the blood-brain barrier. In contrast, systemically applied IL-6 was described by Zalcman et al. [36] to increase the sensitivity to the locomotor-stimulating effects of amphetamine in rats, pointing to a possible impact of peripheral IL-6 upon local CNS activities, either mediated directly or indirectly by the hormonal system. Altogether, IL-6 has achieved considerable attention from neuroscientists as a putative neuroprotective cytokine whose biological effects are highly dependent on its local concentrations available to other cell types [37]. Bioavailability of IL-6 may presumably be further regulated by the intriguing functions of the soluble IL-6 receptor able to act even as an agonist [37]. In summary, it is essential to verify the findings of an NADH-induced IL-6 release by human PBLs, with brain-derived cell types being a more relevant target cell population. Apart from the many biochemical and cellular alterations found in patients affected by neurodegenerative disorders [38, 39], the possible involvement of the immune system has received increasing recognition. Therefore, although multiple functions of NADH in combination with IL-6 may be conceivable in promoting neuronal survival, all these considerations will still remain elusive before the exact mechanism of action of NADH-induced IL-6 production is fully understood, which will be the subject of further investigations.

Acknowledgments

For technical assistance we thank Roswitha Beyer and Carola Vogler. Statistical evaluation of the data was done by Prof. Dr. H. Skarabis, Free University of Berlin, Germany.

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