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A proline-rich polypeptide complex and its nonapeptide fragment inhibit nitric oxide production induced in mice

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Abstract

A proline-rich polypeptide complex (PRP) isolated from ovine colostrum shows immunoregulatory and procognitive activities. It shows beneficial effects in Alzheimer's disease (AD) patients when orally administered in the form of tablets called Colostrinin®. The mechanism of action of PRP/Colostrinin® in AD has not been yet clarified. It is known that oxidative stress and overproduction of NO may enhance neurodegenerative processes. PRP regulates the secretion of cytokines, inhibits NO and O_2^- release in cell cultures. Since the results on isolated cells or cell lines frequently do not reflect the events in vivo, the effect of PRP and its nonapeptide fragment (NP) on the level of NO_2^- in sera of mice untreated or intraperitoneally treated with LPS was studied. PRP and NP did not induce production of NO. However, when applicated 6 h after LPS, they inhibited the release of NO induced by LPS in about 30–50%. The results in vivo presented in this paper confirm the results obtained in cell cultures and indicate that the beneficial effects of PRP/Colostrinin® observed in AD patients may be, among others, due to an inhibition of overproduction of NO.

Keywords: PRP; NP; Inhibitors; Nitrites; In vivo

1. Introduction

A proline-rich polypeptide complex (PRP) isolated from ovine colostrum contains a high proportion of proline residues (25%) and hydrophobic amino acids (40%). It is composed of peptides of various molecular mass up to 3000 Da [1–3]. PRP shows immunoregulatory properties. It induces maturation and differentiation of murine thymocytes and affects humoral and cellular immune responses, both in vivo and in vitro [2,4,5]. The polypeptide is not species specific and is active in mice [4,5], humans [6] and rats [7]. Among biological functions of PRP, regulatory activity on the secretion of an array of cytokines was found by Zabłocka et al. [8]. The net effect of PRP depended on the actual state of the animals studied. PRP seems to restore the balance in cellular immune functions.

In mice, immunoregulatory activity similar to the untreated PRP, showed a nonapeptide fragment (NP): Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro isolated from the chymotrypsin digestion products of PRP [2,9,10].

It was subsequently discovered that PRP has beneficial effect on the cognitive functioning of older rats [7]. PRP showed signs of psychostimulation, improved the mood and cognitive abilities in humans. In the form of orally administered tablets called Colostrinin® containing 100 μg of PRP, it improves the outcome of Alzheimer's disease (AD) patients [6,11,12].

The mechanism of action of PRP/Colostrinin® in AD patients is not yet clarified. Recently, an important role of the immune system in the pathogenesis of neurodegenerative diseases, including AD, was shown [13–18]. Inflammatory processes, oxidative stress and the overproduction of nitric oxide (NO) may enhance the development of AD [19–24]. Our preliminary experiments showed, that PRP inhibits

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the production of superoxide anion and nitric oxide induced by LPS in cultures of human whole blood cells and in murine macrophages [25]. Boldogh et al. [26,27] showed antioxidant effects of PRP in pheochromocytoma PC 12 cell line and in ECV 304 human endothelial cell line.

Results obtained from experiments on isolated cells or cell lines not always reflect the events in vivo, where interplay of cells and many regulatory effectors occur [28]. Therefore, we decided to study the effect of PRP and NP on the level of nitric oxide (NO) induced in sera of mice by intraperitoneal treatment with LPS. The results obtained showed that both, PRP and NP, can inhibit the release of NO. This suggests that inhibition of overproduction of NO might be involved in beneficial effects of PRP/Colostrinin® in AD patients.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, *Escherichia coli*, 055;B5), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), leucoagglutinin (PHA-L), Concanavalin A (Con-A), nitrate reductase, β-NADHP (tetrasodium salt) were obtained from Sigma, USA. *N*-(1-Naphthyl)-ethylenediamine was purchased from Serva Feinbiochemica (Germany). Sulphanilamide, sodium nitrite, and orthophosphoric acid were obtained from the Polish Chemical Reagents, Poland. PRP was prepared from ovine colostrum according to the procedure of Janusz and Lisowski [1,2]. The nonapeptide fragment (NP) of PRP: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro was synthesized by a solid-phase method and kindly provided for our experiments by Professor G. Kupryszewski from the Institute of Chemistry, University of Gdansk, Poland.

2.2. Animals

Seven- to nine-week-old female Balb/c mice were purchased from the Animal Farm of our Institute. The treatment of the laboratory animals and experimental protocols of the present study adhered to the guidelines of the Institute's Authority for Laboratory Animal Care.

2.3. Nitrite/nitrate generation

Mice were lightly anesthetized by ether inhalation and intraperitoneally injected with PRP (0.1, 1.0, 10.0 or 100 μg per mouse) or NP (10.0 or 100.0 μg per mouse) alone or mixed with LPS (15 μg per mouse) for determination of NO induction. All reagents were sterilized and diluted in sterile 0.15 M NaCl to a final volume 0.5 ml. Control mice received sterile 0.15 M NaCl, or only LPS. Blood samples were collected by orbital sinus puncture 1 h or 3, 6, 9, 12 and 24 h after administration of reagents. The blood samples were allowed to clot for 1 h at room temperature and then centrifuged at 700×*g* for 10 min at 4 °C.

2.4. Nitrite/nitrate determination

NO production was measured by estimating the stable NO metabolite, nitrite. The sera obtained were centrifuged at 1500×*g* through 0.22-μm Milipore filters to remove opalescence. The sera were kept frozen until nitrite analysis. Nitrite and nitrate levels, indicators of NO synthesis, were measured in sera after reduction of nitrate to nitrite with NADPH nitrate reductase as described by Guevara et al. [29] and by Moshage et al. [30] with some modifications. In short, 100-μl samples of sera were incubated for 45 min at 37 °C with nitrate reductase (25 mU/sample) in the presence of β-NADPH (final concentration 80 μM) in 20 mM Tris buffer, pH 7.6. The total volume of the reaction mixture was 300 μl. After the enzymatic conversion, nitrite concentration in the sera were measured using Griess reagent. Each serum sample was treated with equal volume of the Griess reagent (0.1% *N*-(1-naphthyl)-ethylenediamine dichydrochloride and 1% sulfanilamide in 5% phosphoric acid). After 10 min incubation at room temperature, the absorbance at 550 nm was measured. The concentration of nitrite was calculated from a $NaNO_2$ standard curve.

3. Results and discussion

To elaborate optimal conditions for experiments, we first studied effects of LPS, Con-A, fMLP, and PHA, administered orally or intraperitoneally on the nitric oxide release in sera of mice. The best results were obtained with LPS which was earlier shown to induce de novo synthesis of iNOS protein [23]. The dose of 15 μg LPS per mouse administered intraperitoneally gave the increase of NO release which was satisfactory for our experiments. Osakabe et al. [31] and TerSteege et al. [32] showed, that the concentration of NO_2^-/NO_3^- in the serum correlates with induction of iNOS mRNA.

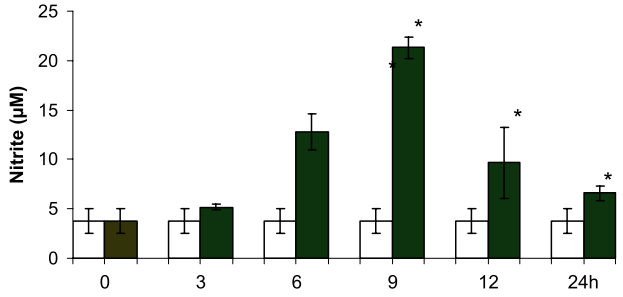

Fig. 1. Effect of LPS on nitric oxide (NO) release in mice. LPS (15 μg per mouse) was administered intraperitoneally to mice. Blood samples were collected directly after administration (0) and 3, 6, 9, 12 or 24 h after LPS administration (■). Control mice (□) received 0.15 M NaCl only. Accumulation of NO_2^-, a stable end product used as an indicator of NO production, was assayed by the Griess reaction. Each bar represents the mean ± S.D. of NO_2^- concentration from three independent experiments performed on 3–4 animals in each experiment (total $n = 9–12$). The data were analysed using Student's *t*-test for independent samples. *$p<0.05$ when compared to control mice.