Pergamon

Life Sciences, Vol. 57, No. 26, pp. PL 401-406, 1995

0024-3205/95 $9.50 + .00

0024-3205(95)02244-9

PHARMACOLOGY LETTERS
Accelerated Communication

5,6-DIHYDROXYINDOLE-2-CARBOXYLIC ACID, A DIFFUSIBLE MELANIN PRECURSOR, IS A POTENT STIMULATOR OF LIPOPOLYSACCHARIDE-INDUCED PRODUCTION OF NITRIC OXIDE BY J774 MACROPHAGES

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(Submitted August 8, 1995; accepted August 15, 1995;
received in final form October 13, 1995)

Abstract. Pre-incubation of J774 murine macrophages with 5,6-dihydroxyindole-2-carboxylic acid (DHICA), a diffusible intermediate in the biosynthesis of eumelanins, leads to a marked increase in the levels of nitric oxide (NO) produced by lipopolysaccharide (LPS)-induced NO-synthase (iNOS). The effect varies with DHICA concentration, being maximum at a concentration of 1 x 10^{-6}M, and is suppressed by the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). No stimulation is observed when macrophages are exposed to DHICA after activation with LPS, indicating that the indole does not affect the catalytic activity of iNOS. These results point to a hitherto unrecognized role of DHICA as a chemical messenger mediating interaction between active melanocytes and macrophages in epidermal inflammatory and immune responses.

Key Words: 5,6-dihydroxyindole-2-carboxylic acid, melanocyte activity, immune system

Introduction

Skin inflammation comprises a very complex scenario of biological and biochemical events which result, *inter alia*, in the activation of macrophages and other inflammatory cells (1). When recruited to a site of injury, macrophages undergo a respiratory burst which is accompanied by the generation of nitric oxide (NO) as part of the inflammatory response (2). Such a process involves oxidation of L-arginine by an inducible, Ca^{2+}/calmodulin-independent isoform of nitric oxide synthase (iNOS) (3). Following stimulation, e.g. by lipopolysaccharide (LPS) and cytokines, the enzyme is expressed over a prolonged period of time and produces relatively large quantities of NO which elicits a range of cytotoxic responses, leading eventually to destruction of microorganisms and tumor cells (4).

In spite of the growing interest in the biochemical mechanisms underlying macrophage activation and NO production, little attention has thus far been paid to a possible interaction between these inflammatory cells and the epidermal pigmentary system, which also plays a central role in skin inflammation (5,6).

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Ample evidence has amassed indicating that cutaneous melanocytes respond dynamically to inflammatory stimuli by increasing the levels of melanin pigments as well as by producing a variety of colorless melanogenic precursors, including 5,6-dihydroxyindoles and 5-S-cysteinyldopa, which are excreted into body fluids (7). Whether and by what mechanism diffusible melanin precursors play a role in the skin inflammatory processes is however unknown. We have now investigated the effect of some melanogenic intermediates on the activity of cultured murine macrophages, and have found that 5,6-dihydroxyindole-2-carboxylic acid (DHICA) is a potent and specific enhancer of NO production by LPS-activated macrophages.

Methods

Materials. L-dopa, 5,6-dimethoxyindole, 5-hydroxyindole-2-carboxylic acid were from Aldrich. 5,6-Dihydroxyindole-2-carboxylic acid and 5-S-cysteinyldopa were prepared by previously described procedures (8,9). 5-Methoxy-6-hydroxyindole-2-carboxylic acid was prepared from commercial 5-methoxy-6-benzyloxyindole-2-carboxylic acid (Sigma) by a catalytic hydrogen transfer procedure: To a solution of 5-methoxy-6-benzyloxyindole-2-carboxylic acid (200 mg) and ammonium formate (200 mg) in methanol (15 ml), 50 mg of 5% Pd on charcoal were added, and the resulting mixture was vigorously stirred. After about 2h the starting material was consumed, as evidenced by thin layer chromatography on silica gel plates. The mixture was evaporated to dryness and the residue was taken up in ethyl acetate, washed with little water and evaporated to give about 80% yield of the product.
Purity was checked by thin layer chromatography and proton magnetic resonance spectroscopy. N^G-monomethyl-L-arginine (L-NMMA) was from Sigma. All reagents for cell culture except foetal calf serum (Flow laboratories) were from Gibco. Lipopolysaccharide (LPS) was extracted from Salmonella typhosa and was purchased from Difco. Statistical analysis was performed by using the two-tailed Student's t-test.

Cell culture. The murine monocyte/macrophage cell line J774 (American Tissue Culture Catalogue T1B 67) was grown in suspension culture in Techne stirrer bottles, spun at 25 rpm and incubated at -95% air for 2h. The cell viability was shown by the trypan blue exclusion test to be > 95%. Cells were incubated in Hank's balanced salts solution with DHICA at different concentrations for 2 h. This time was experimentally determined to be the optimum time to observe enhancement of macrophage activity without appreciable autoxidation of DHICA. The medium containing DHICA was then replaced with DMEM and the cells were activated by LPS (0.1 μg/ml).
In some experiments LPS concentration was varied in the range 0.001-1 μg/ml. The effect of other melanin precursor was assayed as above. In specific inhibition experiments L-NMMA (3.0×10^{-5} M) was added to macrophages pretreated with DHICA (1×10^{-6} M) and LPS (0.1 μg/ml). The stability of DHICA in the culture medium was checked by UV spectrophotometry and HPLC analysis.

Nitric oxide assay. Production of NO by iNOS was assayed by measuring the amount of nitrite in the culture medium by the Griess reaction (10). After 24h incubation, supernatants from the plated cells (400 μl) were mixed with an equal volume of Griess reagent (1% sulphanilamide-0.1% naphthylethylenediamine dihydrochloride-25% phosphoric acid) and incubated at room temperature for 10 min. The absorbance of the solution was then measured at 550 nm. The results are expressed as nmol of nitrite released by 10^6 cells in 24 h. Control experiments showed that DHICA does not affect the Griess test.

Results

The melanin precursors investigated in the present study include dopa, 5-S-cysteinyldopa (CD), 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and 5-methoxy-6-hydroxyindole-2-carboxylic acid (MHICA). Two indole derivatives structurally related to DHICA were also tested, namely 5-hydroxyindole-2-carboxylic acid (HICA) and 5,6-dimethoxyindole (DMI).

Administration of melanin precursor to unstimulated J774 macrophages did not significantly alter the low production of nitrite observed in otherwise untreated control cells (< 1 nmol/10^6 after 24 h; n=8). Pre-incubation of macrophages with DHICA (10^{-8}-10^{-4} M) for 2 h followed by challenge with 0.1 μg/ml LPS resulted however in marked increases in the nitrite levels released in the medium after 24 h, compared to control cells stimulated with 0.1 μg/ml LPS but not pre-exposed to DHICA (nitrite production = 36.5 ± 4.2 nmol/10^6 cells; n=8) (Fig. 1A).

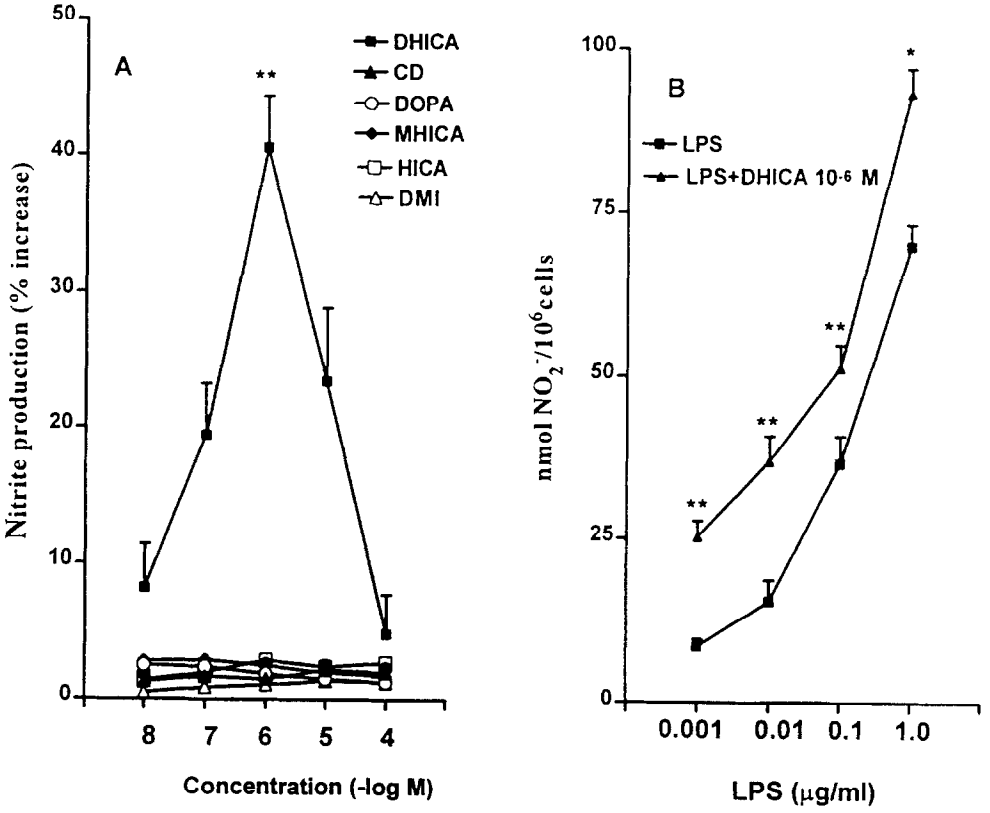

Fig. 1

Effect of pre-incubation with melanin precursors and some analogs on nitrite production by J774 cells stimulated with LPS. (A) Percent enhancement of nitrite production by J774 cells stimulated with LPS (0.1 μg/ml) as a function of compound concentration. Data are calculated as the NO activity of macrophages incubated with various compounds and LPS compared to LPS alone, and represent means ± S.E. of 8 experiments. (B) Nitrite production by J774 cells pre-incubated with 1.0 x 10^{-6} M DHICA and stimulated with various concentrations of LPS. Data represent means ± S.E. of 4-8 experiments. *P<0.05; **P<0.01.

The concentration response curve showed a bell-shaped concentration dependence profile. The maximum enhancing effect (about 40%) was observed with 10^{-6} M DHICA (51.3 ± 3.5 nmol/10^6 cells; n=8). No effect was observed when macrophages were pre-incubated under similar conditions with the other melanin precursors and indole derivatives prior to stimulation with LPS (Fig. 1A). The increase in nitrite production caused by $1.0x10^{-6}$ M DHICA varied significantly with LPS concentration (Fig. 1B). Notably, the lower the LPS concentration, the larger the potentiation effects caused by DHICA. With 1 ng/ml LPS, pre-incubation with DHICA caused an about three-fold increase in nitrite production.

No significant effect was observed when macrophages were exposed to DHICA and other melanin precursors after stimulation with LPS (data not shown). Production of NO_2^- by macrophages activated with 0.1 μg/ml LPS was partially suppressed by the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), irrespective of whether cells had been pre-incubated with $1.0x10^{-6}$ M DHICA or not (Fig. 2). In control experiments, DHICA was found to be fairly stable in the culture medium at a concentration of $1.0x10^{-6}$ M or higher over a period of 2h, indicating that the stimulatory effect on macrophage activity is indeed due to the indole and not to an oxidation product. At lower concentrations, however, autoxidation of the indole became significant after shorter periods of time. The instability of DHICA to autoxidation prevented investigation of the effect of the indole when co-incubated with LPS for 24 h.

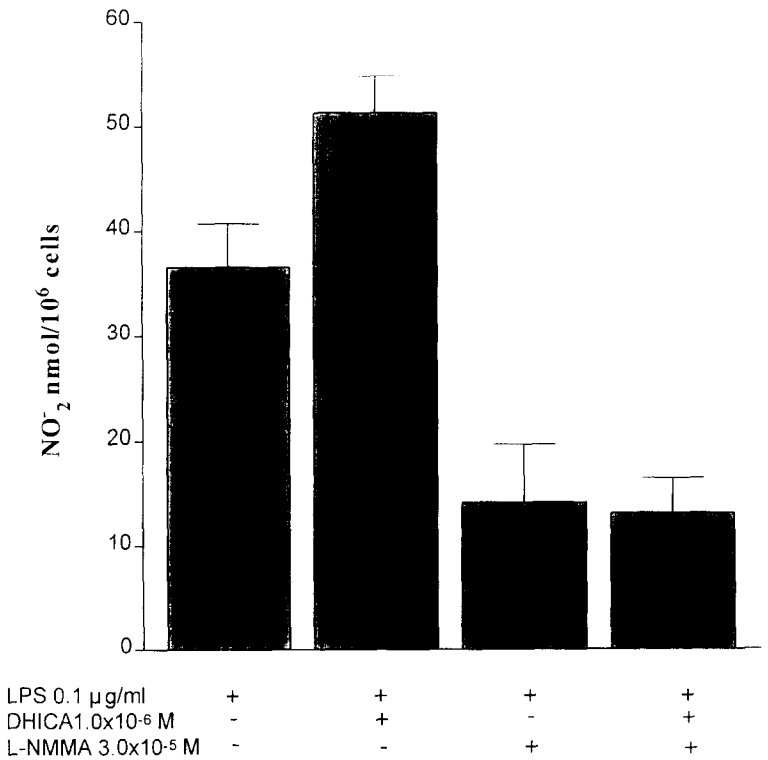

Fig. 2

Inhibitory effect of L-NMMA (30 μM) on nitrite production by J774 cells stimulated with LPS (0.1 μg/ml) with or without pre-incubation with DHICA (1.0×10^{-6} M). Data represent means ± S.E. of 4-8 experiments.

Discussion.

Interest in colorless products of melanocyte metabolism has traditionally been focused on their role as pigment precursors or as metabolic markers of melanoma progression (11). That melanin precursors may have a significance *per se* is however suggested by recent studies showing that 5,6-dihydroxyindole, a major intermediate in the eumelanin pathway, exerts an inhibitory effect on enzymatically-induced lipid peroxidation (12). A similar effect has also been reported on lipid peroxidation in UV-irradiated liposomes (13).

The finding that DHICA, another diffusible melanin precursor, markedly augments the production of NO by LPS-activated murine macrophages would open a new important entry into the role of functionally active melanocytes within the skin immune system (14). Under conditions of increased melanogenesis, e.g. following exposure to inflammatory stimuli, relatively high levels of DHICA may be produced by the action of the enzyme dopachrome tautomerase on dopachrome (15). The indole may then diffuse out of melanocytes to target surrounding macrophages and prepare those cells to produce much more of the cytotoxic mediator NO when they are subsequently exposed to LPS. Such a mechanism could be regarded as part of a more complex interaction between melanocytes and macrophages. Noteworthy, the generation of hybrids between transformed malignant melanocytes and macrophages has recently been suggested to initiate highly aggressive melanoma metastases (16).

The potentiation effect of DHICA is reminiscent of that of several important physiological substances, e.g. interferon-γ(17) and ATP (18). Like these latter, DHICA alone can not induce iNOS gene expression, as indicated from the inability to induce any NO production in the absence of LPS. It also does not influence the enzyme activity, consistent with the lack of effect after macrophage stimulation with LPS. Thus, the likelihood is great that DHICA enhances the induction of iNOS by acting through the signaling cascade activated by LPS. How this occurs is difficult to assess because of the lack of direct experimental evidence and the currently incomplete knowledge of the mechanisms of activation of macrophages by LPS.

The sharp, approximately bell-shaped concentration-dependence profile of the potentiation effect (Fig. 1A) is probably a reflection of a partial autoxidative decomposition of DHICA in the low concentration range, and of weak cytotoxic effects at near-millimolar doses (19). Other effects, however, which would derive from interaction of DHICA with specific binding sites on the macrophage surface, can not be ruled out at present. In this connection, the recent demonstration (20) that iron starvation potentiates the effect of LPS and other cytokines on the induction of iNOS could be taken to invoke iron chelation as a possible mechanism underlying the observed stimulating properties of DHICA. This view would be consistent with the lack of activity of the DHICA analogs 5-methoxy-6-hydroxyindole-2-carboxylic acid and 5-hydroxyindole-2-carboxylic acid, which are much poorer metal chelators.

Work is in progress to elucidate the molecular events underlying the potentiating effect of DHICA on NO production by LPS-stimulated macrophages, and to assess the biological relevance of this effect to the skin inflammatory response.

Acknowledgments

This work was supported in part by grants from Consiglio Nazionale delle Ricerche and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Rome). We are indebted to Professors G. Prota and M. Di Rosa for their encouragement and helpful discussions.

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