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Effect of picolinic acid on nitric oxide synthesis in murine macrophage

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Abstract

To determine the effect of picolinic acid on NO· production, murine macrophages were incubated with either medium, various concentrations of picolinic acid, or IFN-γ plus picolinic acid for 48 hr. Picolinic acid does not induce NO· production by itself, it acted synergistically with INF-γ for the induction of reactive nitrogen intermediate production in murine macrophages. Thymidine incorporation appeared to be reciprocally related to nitrite levels, suggesting that IFN-γ plus picolinic acid induced NO· synthesis exerted antiproliferative effects.

Key words : Picolinic acid, Interferon-gamma, Nitric oxide

Introduction

Nitric oxide(NO·) has been identified as a major effector molecule, involved in the expression of tumoricidal and microbicidal activities exerted by activated murine macrophages[1~4]. The production of NO·, from the L-arginine metabolic pathway, is controlled in murine macrophages by an inducible enzyme, nitric oxide synthase (NOS)[1,5]. The macrophage NOS is inducible by INF-γ in combination with several agents, including LPS, TNF, and IL-2[6~10]. Although much information has been accumulated regarding the role of NOS in the production of NO· by activated murine macrophages, the molecular mechanism underlying this process are still poorly understood.

Recent studies have suggested that amino acid catabolites may be important for the activation and expression of effector functions by murine macrophages[3,11,12]. Picolinic acid, a terminal metabolite of L-tryptophan, is a potent co-stimulatory agent for the induction of tumoricidal activity in mouse

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peritoneal macrophages[13]. The metabolism of two amino acids, L-arginine and L-tryptophan, has been associated with the tumoricidal and microbicidal activities of murine mononuclear phagocytes[14~17]. Therefore these two amino acids can be considered to be physiologic macrophage stimuli that participate in the activation and the expression of macrophage effector function[18]. However, the relationship between L-arginine and L-tryptophan degradation products and macrophage activation has not been established[18]. The purpose of this study was to investigate whether picolinic acid is a co-stimulus for the induction of L-arginine-dependent reactive nitrogen intermediate production in murine macrophages.

Materials and Methods

Isolation of macrophages from ascites

Macrophages were obtained from BALB/c mice injected with 2㎖ of sterile 3% thioglycollate(Difco Laboratories, Detroit, MI). Four days after injection, peritoneal exudate cells were harvested by peritoneal lavage with 6㎖ of ice-cold phosphate-buffered saline(PBS)/mouse. These cells were washed twice and then suspended at 2×10^6 cells/㎖ in RPMI 1640 medium supplemented with 5% fetal calf serum(Hyclone Laboratories, Inc, Logan, UT), 100 units/㎖ penicillin G (Sigma Chemical Co, St Louis, MO), 50㎎/㎖ streptomycin(Sigma), and 2mM glutamine (Sigma, working medium). After a 2hr incubation in 75-cm^2 culture flasks(a final volume of 20 ㎖l/flask, Becton Dickinson Labware, Oxnard, CA) at 37℃ in a humidified 95% air/5% CO_2 atmosphere, non-adherent cells were removed by repeated washing with ice-cold PBS. Adherent cells (peritoneal exudate macrophages, PEM) were harvested using a cell scraper(Baxter S/P brand diSPo cell scraper, Baxter Health Care Corporation, McGraw Park, IL) and re-suspended at 10^6 cells/㎖ in working medium for further experiments.

Reagents

Recombinant mouse IFN-γ was purchased from Genzyme(Cambridge, MA). Picolinic acid was purchased from Sigma(purity approximately 99%). Picolinic acid was dissolved in Hanks' balanced salt solution (without Ca^{2+}/Mg^{2+}, Flow Laboratories, McLean, VA, USA) and the pH was adjusted to 7.4. The stock solution was then passed through a 0.2㎛ filter, aliquoted and stored at -20℃. During the course of the experiments, all reagents and media were tested by *Limulus amebocyte* lysate assay (detection limit 10pg/㎖: Whittaker Bio-products, Walkersville, MD) to exclude potential endotoxin contamination.

Analysis of Nitric Oxide synthesis by macrophages

Macrophages were cultured in working medium(2×10^5 cells/well) in 96-well flat-bottom plates(at a final volume 200㎕/well). In some experiments, IFN-γ (25U/㎖) and/or picolinic acid(0.5-10.0 mM) were added at the onset of the culture. After a 48 hr incubation at 37℃ in humidified 95% air/5% CO_2 atmosphere, nitrite, a stable metabolite of NO·, was quantified in culture supernatants using a previously described colorimetric microtiter assay[19]. Briefly, 50㎕ aliquots of the culture supernatants dispensed into 96-well-flat bottom microtiter plates (Nunc, Roskilde, Denmark) were incubated with 100㎕ of a 1 : 1 mixture of 1% sul-

fanilamide(Sigma) in 30% acetic acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride(Sigma) in 60% acetic acid at room temperature. After 5 min, absorbance was measured at 570㎚ using a microtiter plate reader(MR700, Dynatech Laboratories Inc, Alexandria, VA). Concentrations were determined from a linear standard curve generated from serial concentrations(6.25 to 200 μM) of sodium nitrite(Sigma) in working medium. Results of triplicate measurements were presented as mean ± SD.

Effect of nitric oxide on cell proliferation

Microtiter wells containing 2×10^5 macrophages in working medium were cultured in the presence of IFN-γ(25U/㎖) and varying concentrations of picolinic acid(0.5-10.0 mM) for 30 hr and then pulsed with tritiated thymidine(0.5 μCi/well [^{3}H]TdR, 2.0 Ci/㎜ol; DuPont NEN, Boston, MA) for an additional 18 hr(total 48 hr), maintaining a constant final volume of 200$\mu\ell$/well. Following harvesting onto glass fiber filters using a PhD cell harvester(Cambridge Technology, Cambridge, MA), samples were suspended in Optifluor scintillation fluid(Packard, Downers Grove, IL) and the incorporated [^{3}H]TdR was measured in a Packard Tricarb 1500 scintillation counter(Packard)[20]. Each assay was performed at least in triplicate, and the results were expressed as a mean ± SD.

Results

To determine the effect of picolinic acid on NO· production, murine macrophages were incubated with either medium, various concentrations of picolinic acid, or IFN-γ plus picolinic acid for 48 hr. IFN-γ(25U/㎖) alone and picolinic acid(0.5-10mM) alone did not increase NO· accumulation above control levels. However, Nitrite production in IFN-γ(25U/㎖) plus picolinic acid was found to be increased in a dose-dependent manner of picolinic acid. Picolinic acid acted as a costimulus with IFN-γ(25U/㎖) to induce NO·, and induced its maximal effect with 6-8 mM of picolinic acid(Fig 1). This result suggested that picolinic acid is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages.

One of the major effects of NO· on target cells is inhibition of ribonucleotide reductase, the rate-limiting step in de novo DNA synthesis[20]. To determine whether IFN-γ plus picolinic acid induced NO· synthesis inhibited cell proliferation in macrophages, we measured the effect of increasing concentrations of picolinic acid on thymidine incorporation into DNA in macrophages. This experiment demonstrated that thymidine incorporation appeared to be reciprocally related to nitrite levels, suggesting that IFN-γ plus picolinic acid induced NO· synthesis exerted antiproliferative effects(Fig 2).

Discussion

Because picolinic acid is a natural product derived from L-tryptophan catabolism, it is reasonable to suggest that picolinic acid may be a physiologic costimulus for reactive nitrogen intermediate production[18]. Picolinic acid is described as a terminal catabolite of tryptophan[11,18]. However, the possibility of its further metabolism by macrophages can not be ruled out[18]. The production of NO·, from the L-arginine metabolic pathway, is controlled in murine macrophages by an inducible enzyme, nitric oxide synthase(NOS)[1,5]. The macrophage NOS is inducible by INF-γ

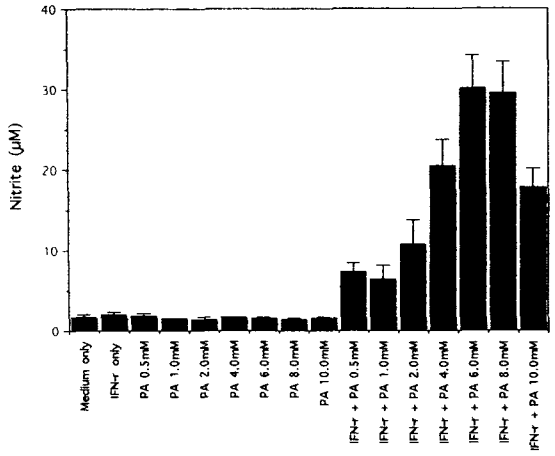

Fig 1. Effect of picolinic acid on NO· synthesis in murine macrophages.
Murine macrophages(2×10^5 cells/well) were incubated with the indicated concentrations of picolinic acid and/or IFN-γ(25U/mℓ) for 48 hr. Nitrite(NO_2^-, a stable metabolite of NO·) was measured in culture supernatant by colorimetric assay. The results are expressed as the mean±SD of nitrite concentration(μM) produced by triplicate wells.

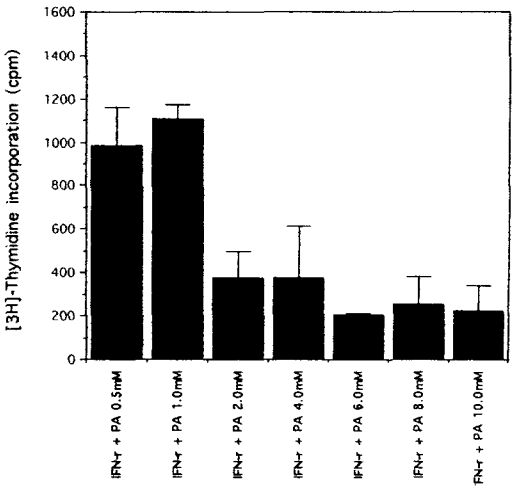

Fig 2. Effect of picolinic acid on [^{3}H]-Thymidine incorporation.
Murine macrophages(2×10^5 cells/well) were cultured with the indicated concentrations of picolinic acid and IFN-γ(25U/mℓ) for 30 hr, and then pulsed with tritiated thymidine(0.5 μCi/well) for additional 18 hr(total 48 hr). Cells were harvested onto glass fiber filters and analyzed for thymidine incorporation into DNA scintillation counting. Results are mean±SD of triplicate wells.

in combination with several agents, including LPS, TNF, and IL-2[6~10]. As opposed to IL-2 or TNF, LPS can also induce NO· production alone when used at higher concentration[18]. We have studied the effects of picolinic acid on the production of reactive nitrogen intermediate by murine macrophages. To determine the effect of picolinic acid on NO· production, murine macrophages were incubated with either medium, various concentrations of picolinic acid, or IFN-γ plus picolinic acid for 48h. IFN-γ alone and picolinic acid alone did not increase NO· accumulation above control levels. However, Nitrite production in IFN-γ(25 U/mℓ) plus picolinic acid was found to be increased in a dose-dependent manner of picolinic acid. Picolinic acid acted as a costimulus with IFN-γ to induce NO·, and induced its maximal effect with 6~8 mM of picolinic acid. This result suggested that although picolinic acid does not induce NO· production by itself, it acted synergistically with INF-γ for the induction of reactive nitrogen intermediate production in murine macrophages and amino acid catabolites may be important for the activation by macrophages.

NO· is known to exert antiproliferative effects on target cells by inhibiting ribonucleotide reductase, the rate-limiting step in DNA synthesis[20]. To evaluated whether IFN-γ plus picolinic acid induced NO· synthesis inhibited cell proliferation in murine macrophages, we measured the effect

of increasing concentrations of picolinic acid on thymidine incorporation into DNA in macrophages. This experiment demonstrated that thymidine incorporation appeared to be reciprocally related to nitrite levels, suggesting that IFN-γ plus picolinic acid induced NO· synthesis exerted antiproliferative effects.

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