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Enhancement of inducible-type NO synthase gene transcription by protein synthesis inhibitors

Activation of an intracellular signal transduction pathway by low concentrations of cycloheximide

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Abstract

Treatment of mouse macrophage-like RAW 264 7 cells with certain protein synthesis inhibitors is followed by accumulation of the mRNA for the inducible isoform of nitric oxide synthase (i-NOS) The activity of these compounds on the i-NOS gene in RAW 264 7 cells was analyzed here in detail Results show that both cycloheximide and anisomycin can efficiently induce i-NOS mRNA, even when used at concentrations so low (0 25 μg/ml) to have only negligible effects on protein synthesis, puromycin, on the other hand, shows only a limited effect on i-NOS mRNA expression, detectable only when cells are treated with higher concentrations of inhibitor (25 μg/ml) In RAW 264 7 cells, low concentrations of cycloheximide trigger an immediate-early gene response, as indicated by induction of c-*fos* and *JE* mRNAs, and can efficiently activate transcription of transiently transfected recombinant reporter genes including either the i-NOS or the c-*fos* gene promoters

Key words Nitric oxide synthase, Transcription, Cycloheximide, Anisomycin, Signal transduction, Phosphorylation

1. Introduction

Cytokines, inflammatory peptides and components of the bacterial cell wall control the rate of nitric oxide (NO) production by macrophages, as well as numerous other mesenchymal or epithelial cell types, by affecting inducible-type nitric oxide synthase activity (i-NOS) [1–2] This is achieved primarily by changes of i-NOS gene transcription, as demonstrated for bacterial lipolysaccharides (LPSs) and interferon gamma (IFN-γ), the best characterized inducers of i-NOS activity in macrophages [3–5]. Central to our further understanding of the NO role in key biological processes, such as inflammation and defense against invading microorganisms and tumor cells, is thus a better knowledge of the pathways that control i-NOS gene expression Treatment of RAW 264 7 cells with certain protein synthesis inhibitors results in rapid and significant accumulation of i-NOS mRNA [5] Exposure of cell cultures to 25 μg/ml cycloheximide or anisomycin for 5–6 h, a condition that reduces protein synthesis by more than 90%, is followed by a 2 5- to 5-fold increase of i-NOS mRNA concentration in the cells, puromycin, although equally effective in inhibiting protein synthesis, is much less efficient in determining i-NOS mRNA expression [5] These differences suggested to us that the stimulatory activity of cycloheximide and anisomycin on i-NOS mRNA expression derived not from inhibition of protein synthesis, but instead from a distinct action of these compounds within the cell Recent findings, suggesting pleiotropic activities of cycloheximide and anisomycin [6–8], that include agonist-like effects on signal transduction pathways both in macrophages [9–10] and other cell types [11–14], prompted us to investigate in more detail regulation of i-NOS gene expression by protein synthesis inhibitors

Results show that cycloheximide and anisomycin, at concentrations as low as 0 25 μg/ml, significantly increase steady-state i-NOS mRNA levels in RAW 264 7 cells; puromycin was much less effective in inducing i-NOS mRNA, and did so only when used at higher concentrations (25 μg/ml) Kinetic analysis of i-NOS mRNA accumulation in response to a low dose of cycloheximide, and comparison with c-*fos* and *JE* mRNA accumulation under the same conditions, suggest that

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the i-NOS gene is not a classical 'immediate-early' gene [15]. Furthermore, low doses of cycloheximide were found to activate the mouse i-NOS gene promoter cloned upstream of the bacterial CAT reporter gene and transiently transfected in RAW 264.7 cells

These data indicate that i-NOS gene transcription in RAW 264 7 macrophages is controlled by the effector(s) of at least one signal transduction pathway, that is sensitive to low concentrations of cycloheximide.

2. Materials and methods

2 1 Reagents and cell culture

The mouse macrophage-like cell line RAW 264 7 (ATCC TIB71) was obtained from the American Type Culture Collection (ATCC, USA) Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, USA), supplemented with 2 mM L-glutamine, antibiotics (50 U/ml penicillin-G, 50 μg/ml streptomycin and 125 ng/ml amphotericin B, Sigma, USA) and 10% fetal bovine serum (MultiSer, Cytosystems, Australia) Bacterial lipopolysaccharide (LPS, from *E coli* serotype 0127 B8), cycloheximide, puromycin and anisomycin were from Sigma, recombinant mouse IFN-γ was a generous gift of Shionogi Pharmaceuticals Company (Japan)

2 2 RNA purification, electrophoresis and transfer to nylon membranes

Treatment of RAW 264 7 cells with inducers, and cellular RNA extraction were performed as described earlier [5] Twenty-five μg of total RNA was electrophoresed in 1% formaldehyde-agarose gels according to standard protocols [16], blotted onto nylon filters (Hybond-N, Amersham, USA) by capillary transfer and crosslinked to the membrane by UV irradiation

2 3 Hybridization of membranes with ^{32}P-labelled DNA probes

RNA-containing filters were hybridized with labelled DNA probes as described [5] Probes used were a 700 bp DNA fragment including the 5′ portion of the cloned rat liver inducible-type NO synthase cDNA (i-NOS probe) [17], the full length mouse c-*fos* [18] and *JE* [19] cDNAs and a 1300 bp DNA fragment including the 3′-untranslated and flanking regions of the rat β-actin gene [20], they were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, DuPont-New England Nuclear, USA) by random priming (Multiprime DNA Labeling System, Amersham) at a specific activity of ca 10^9 cpm/μg DNA Autoradiography was performed by exposing the filters for 12 to 16 h to imaging plates and visualization of the autoradiographic signals on a Fuji Image Analyzer

2 4 Transient transfection and CAT assay

For transient transfections, RAW 264 7 cells were plated on Day 1 at about 10–20% confluency in DMEM, 10% FCS culture medium On Day 2, fresh medium was added and cells were transfected, using the calcium phosphate-DNA co-precipitation method [21] with 5 μg piNOS CAT 1 [5] or pFC4-BL [22] DNA, 3 μg of the β-galactosidase expression vector pnlsLAC-Z (containing the bacterial *LAC-Z* ORF fused to the nuclear localization signal of SV40 large T antigen) [22], as an internal control for transfection efficiency, and carrier DNA (Bluescribe M13+) up to 15 μg total DNA/cell culture dish After 18–20 h, cells were washed once for 30 min with PBS 1× before addition of fresh medium and incubation for an additional 24 h Where indicated, 10^2 U/ml IFN-γ, 5 μg/ml LPS or protein synthesis inhibitors were added to the cell culture media starting from Day 2

CAT enzyme assays were performed in whole-cell extracts as described earlier [5,22], after normalization for β-galactosidase activity and under assay conditions where substrate conversion was within a linear range in respect to the concentration of cell extract used Chloramphenicol acetyltransferase activity was calculated as cpm acetylated chloramphenicol/h/unit βGAL

3. Results and discussion

We have previously shown that cycloheximide, anisomycin and puromycin can induce significant accumulation of i-NOS mRNA in RAW 264.7 macrophages [5]. Although at the concentration used in those experiments (25 μg/ml) protein synthesis was efficiently inhibited in all cases, substantial differences were observed between the ability of different compounds to determine i-NOS mRNA accumulation in RAW 264.7 cells. cycloheximide and anisomycin were in fact very efficient when compared to puromycin [5] This observation suggested to us the possibility that the action exerted on i-NOS mRNA expression by cycloheximide and anisomycin could only in part depend upon their ability to inhibit protein synthesis

To further investigate this phenomenon, we performed a dose–response experiment, where we measured steady-state i-NOS mRNA levels in RAW 264 7 cells before and after exposure for 5 h to variable concentrations of cycloheximide, puromycin or anisomycin, ranging from 0 25 to 25 μg/ml At the lowest concentration, none of the compounds significantly inhibited protein synthesis in the cells [12–13, and data not shown] Results of a representative experiment are reported in Fig. 1A, and show that cycloheximide or anisomycin can increase significantly i-NOS mRNA levels in RAW 264.7 cells already at 0.25 μg/ml (3- to 6-fold and 4- to 8-fold induction, respectively· compare lane 1 with lane 2 or lane 8). In contrast, puromycin was ineffective (lane 5), even when used at a 10-fold higher concentration (2.5 μg/ml lane 6); a limited effect on i-NOS mRNA was observed only with 25 μg/ml of this inhibitor (1.5- to 3-fold induction· lane 7), coincident with its maximal inhibitory effect on protein synthesis Higher concentrations of either compound (up to 125 μg/ml) did not increase i-NOS mRNA expression above the value detectable when 25 μg/ml was used (data not shown) In Fig 1A are also reported the effects of combined stimulation of the cells with LPS and IFN-γ (lane 11) to show how i-NOS mRNA accumulation in response to low doses of cycloheximide or anisomycin reaches much lower levels than those occurring in response to these two physiological inducers (8- to 10-fold lower compare lanes 2 to 4 or 8 to 10 with lane 11)

The activity of cycloheximide was then analysed in more detail by performing a kinetic analysis of i-NOS mRNA accumulation in response to a low concentration of the inhibitor. Cycloheximide was selected since it has been shown to exert in macrophages important effects both on gene expression [9–10] and on the activity of NF-κB transcription factors [10]. Cells were harvested immediately before (time 0) or at the indicated times after addition in the culture medium of 0 25 μg/ml cycloheximide. As a control, one of the cultures was treated for 5 h with 5 μg/ml LPS. i-NOS mRNA levels were then assessed in 25 μg total cellular RNA by Northern blot hybridization (lower panel in Fig 1B). Maximal i-NOS mRNA accumulation was detected after 5 h of cyclo-

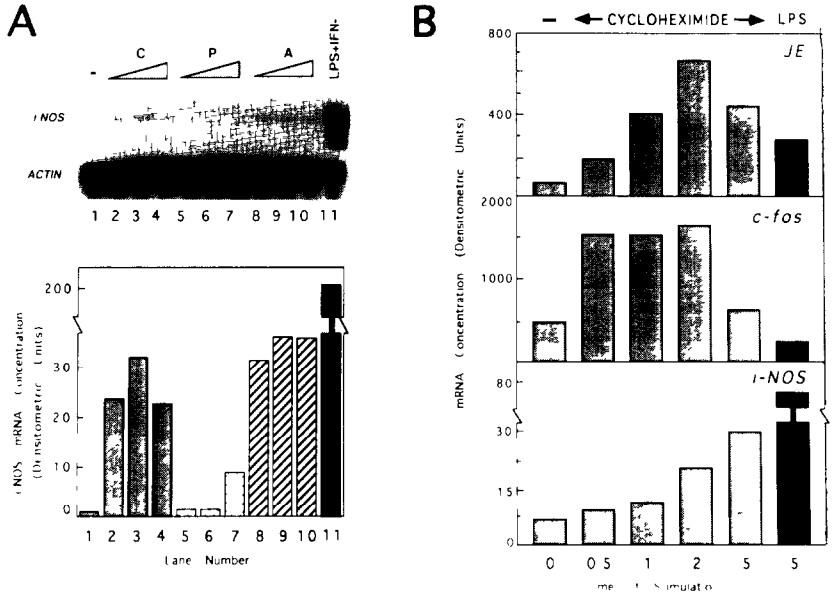

Fig 1 Activation of gene expression following exposure of RAW 264 7 macrophages to low concentrations of protein synthesis inhibitors
(A) Dose-response analysis of i-NOS mRNA induction by cycloheximide, puromycin and anisomycin Cells were incubated for 5 h either in medium alone (lane 1), or in medium supplemented with cycloheximide (C, 0 25, 2 5 or 25 μg/ml lanes 2 to 4), puromycin (P, 0 25, 2 5 or 25 μg/ml lanes 5 to 7), anisomycin (A, 0 25, 2 5 or 25 μg/ml lanes 8 to 10) or bacterial lipopolisaccharide (LPS, 5 μg/ml) plus mouse interferon gamma (IFN-γ, 100 U/ml lane 11) Following cell lysis and RNA purification, i-NOS (i-NOS) and β-actin (ACTIN) mRNA concentration was assessed in 25 μg total cellular RNA by 'Northern blot' analysis Data are reported as actual autoradiograph of a representative filter (*top*) or as graphic representation of the results obtained by quantitative densitometric analysis of autoradiographic signals corresponding to the i-NOS mRNA, assessed in multiple, independent experiments and corrected on the basis of β-actin mRNA concentration in the same lane (*bottom*)
(B) Kinetics of JE, c-fos and i-NOS mRNA induction by cycloheximide Cells were incubated for the indicated times in medium supplemented with 0 25 μg/ml cycloheximide, or for 5 h in medium containing 5 μg/ml LPS Following cell lysis and RNA purification, *JE*, c-*fos* and i-NOS (i-NOS) mRNA concentration was assessed in 25 μg total cellular RNA by 'Northern blot' analysis Data are reported as graphic representation of the results obtained by quantitative densitometric analysis of autoradiographic signals corresponding to the each mRNA, corrected on the basis of the β-actin mRNA hybridization signal detected in the same lane

heximide treatment, the longest stimulation time allowed in the experiment shown in the figure (4- to 6-fold increase); extending the length of the stimulation up to 20 h did not result in further increase of i-NOS mRNA levels (data not shown) This gene thus is not a 'primary response' gene, as far as cycloheximide is concerned in RAW 264.7 macrophages whereas it is here directly stimulated by both LPS and IFN-γ [5] iNOS mRNA level rose higher in the cells after LPS stimulation than after cycloheximide treatment (3- to 4-fold higher compare the last two lanes at the bottom of Fig 1B) The same blots were then hybridized with DNA probes specific for *JE* and c-*fos*, two immediate-early genes that, as such, are both inducible and super-inducible by this protein synthesis inhibitor in several cell lines [14, 23] Even these genes are inducible in RAW 264 7 cells by cycloheximide, although their response is more rapid and readily reversible when compared to that of the i-NOS gene (upper panels in Fig 1B) These results show two distinct features of the response of these genes to a low dose of cycloheximide that distinguishes it from that of the i-NOS gene (i) c-*fos* and *JE* mRNA accumulation occurred very early after the beginning of the treatment (maximal within the first 30 min for c-*fos* and between 1 and 2 h for *JE*), and (ii) it was transient, in both cases declining from a peak value within 5 h According to the criteria established for defining immediate-early genes (rapid and transient response to cell stimulation and induction and super-induction by protein synthesis inhibitors) [14], the i-NOS gene is thus not to be classified as an immediate-early gene, despite its response to cycloheximide It is worth mentioning that accumulation of i-NOS mRNA in response to cycloheximide and anisomycin is rather poor when compared to that of certain immediate-early gene mRNAs [9,12,14] A combination of two distinct factors helps to explain this result first, i-NOS mRNA stability is not greatly affected by the translational inhibitors in RAW 264 7 cells (data not show), whereas this is a major factor for immediate-early gene mRNA accumulation during protein synthesis inhibition in a variety of cell types [5] second, RAW 264 7 cells also c-*fos* mRNA accumulation is rather limited (2-to 4-fold, see Fig 1B), to indicate that under these experimental conditions a considerable effect of the inhibitors on mRNA levels it is not to be expected

The results of these experiments are strongly indicative

of a stimulation of i-NOS gene expression by cycloheximide that is independent from its ability to interfere with protein synthesis. When actinomycin D, an inhibitor of RNA synthesis, was used in conjunction with cycloheximide, accumulation of i-NOS mRNA in RAW 264.7 cells was abolished, suggesting that increased synthesis of this RNA, more than a reduction of its degradation rate, was the most likely mechanism involved (data not shown) For this reason, and taking advantage of the fact that low concentrations of this compound do not affect significantly mRNA translation, we transfected the mouse i-NOS gene promoter-CAT reporter gene piNOS CAT1 [5] in RAW 264.7 cells and asked whether cycloheximide treatment was accompanied by increased activity of the i-NOS gene promoter. Results, reported in Fig. 2, demonstrate that in the presence of 0 25 μg/ml cycloheximide transcription of iNOS CAT1 is increased 4- to 8-fold in this macrophage cell line (compare lane 2 with lane 1). For comparison, parallel cultures were transfected with the CAT reporter pFC4-BL [22], which includes the promoter and enhancer of the human c-*fos* gene, responsive to the nuclear effectors of different signal transduction pathways, and also activated by cycloheximide [11]. Even pFC4-BL transcription was enhanced significantly by cycloheximide (2- to 4-fold compare lanes 9 to 11 with lane 8), confirming that low concentrations of this compound activate in RAW 264 7 cells pathways that can lead to increased transcription of certain genes, including also the i-NOS and c-*fos* genes

In conclusion, we have shown here that in macrophages i-NOS gene transcription is positively regulated by one, or more, pathways that are also sensitive to the agonistic activity of low concentrations of certain protein synthesis inhibitors A distinct order of potency of these compounds toward induction of i-NOS mRNA can be clearly seen, namely cycloheximide = anisomycin >> puromycin, independent from their intrinsic ability to inhibit protein synthesis but surprisingly overlapping with their ability to stimulate c-*fos*, c-*jun* and pp33/histone H3 phosphorylation in mouse fibroblasts [14] In view of the stimulatory effects of cyclohexeximide on NF-κB transcription factor activity in murine macrophages [10], and of the likely involvement of NF-κB response elements on both basal and inducible transcription of the i-NOS gene in RAW 264.7 cells ([5,24] and Weisz, A , Cicatiello, L. and Esumi, H , manuscript in preparation), we would like to suggest that the effects of cycloheximide described here is, at least in part, mediated by activation of certain components of this complex that, in turn, leads to more efficient transcription of the i-NOS gene Alternatively, it is also possible that incompletely blocked protein synthesis allows translation of mRNAs encoding newly induced transcription factor(s), so that i-NOS mRNA accumulation is a secondary, rather than primary, response to cycloheximide- and anisomycin-regulated signals in the nucleus

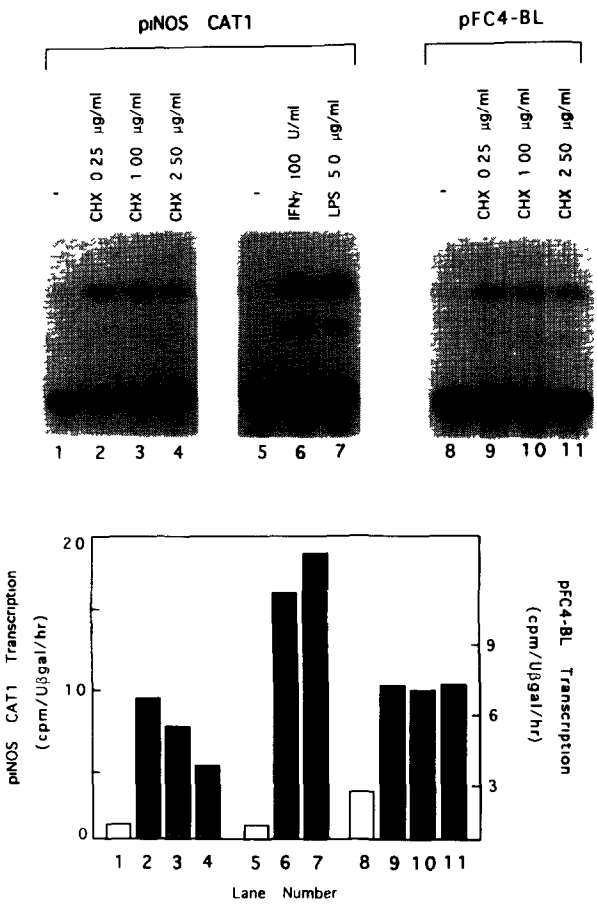

Fig 2 Activation of the cloned mouse i-NOS gene promoter transfected in RAW 264 7 macrophages by low concentrations of cycloheximide pi-NOS CAT1 reporter plasmid (5 μg) was transfected in RAW 264 7 cells by the calcium phosphate-DNA co-precipitation method Cells were then incubated for 24 h in medium alone (lanes 1 and 5), or in medium supplemented with the indicated amounts of cycloheximide (lanes 2 to 4), IFN-γ (lane 6) or LPS (lane 7) Alternatively, 5 μg pFC4-BL, including the human c-*fos* gene promoter and enhancer linked to CAT, were transfected and cells were exposed for 24 h to the indicated amounts of cycloheximide (lanes 8 to 11) CAT activity was measured in whole cell extracts, corrected for differences in transfection efficiency by normalizing the amount of extract used in each case to its content in β-galactosidase internal control Data are reported as actual autoradiograph of a chromatographic plate (upper), or as graphic representation of the results obtained by assessing the amount of acetylated chloramphenicol present in each sample (lower)

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References

[1] Lowenstein, C J and Snyder, S H (1992) Cell 70, 700–707
[2] Knowles, R G and Moncada, S (1992) Trends Biochem Sci 17, 399–402
[3] Lorsbach, R B , Murphy, W J , Lowenstein, C J , Snyder, S H and Russell, S W (1992) J Biol Chem 268, 1908–1913
[4] Xie, Q -W , Whisnant, R and Nathan, C (1993) J Exp Med 177, 1779–1784

[5] Weisz, A., Oguchi, S., Cicatiello, L. and Esumi, H. (1993) J. Biol. Chem., in press
[6] Hua, J. and Hod, Y. (1992) Mol. Endocrinol. 6, 1418–1424
[7] Smalheiser, N. and Rossulek, M. (1992) Int. J. Dev. Neurosci. 10, 467–472
[8] Hayes, N., Biswas, C., Strout, H.V. and Berger, J. (1993) Biochem. Biophys. Res. Commun. 190, 881–887
[9] Farber, J.M. (1992) Mol. Cell. Biol. 12, 1535–1545
[10] Collart, M.A., Bauerle, P. and Vassali, P. (1990) Mol. Cell. Biol. 10, 1498–1506
[11] Subramaniam, M., Schmidt, L.J., Crutchfield III, E.C., and Getz, M.J. (1989) Nature 340, 64–66
[12] Mahadevan, L.C. and Edwards, D.R. (1991) Nature 349, 747–748
[13] Mahadevan, L.C., Willis, A.C. and Barrat, M.J. (1991) Cell 65, 775–783
[14] Edwards, D.R. and Mahadevan, L.C. (1992) EMBO J. 11, 2415–2424
[15] Herschman, H.R. (1991) Annu. Rev. Biochem. 60, 281–319
[16] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1993) Current Protocols in Molecular Biology, John Wiley, New York
[17] Adachi, H., Iida, S., Oguchi, S., Ohshima, H., Suzuki, H., Nagasaki, K., Kawasaki, H., Sugimura, T. and Esumi, H. (1993) Eur. J. Biochem. 217,37–43
[18] Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E., and Leder, P. (1988) Cell 55, 917–924
[19] Cochran, B.H., Reffel, A.C. and Stiles, C.D. (1983) Cell 33, 939–947
[20] Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. and Jaffe, D. (1983) Nucleic Acids Res. 11, 1759–1771
[21] Gorman, C. (1986) In: DNA Cloning – a Practical Approach (Glover D.M., Ed.), Vol. 2, pp. 143–190, IRL Press, Oxford
[22] Weisz, A., Rosales, R. (1990) Nucleic Acids Res. 18, 5097–5105
[23] Cicatiello, L., Sica, V., Bresciani, F. and Weisz, A. (1993) Receptor 3, 17–30
[24] Xie, Q.-W., Whisnant, R. and Nathans, C. (1993) J. Exp. Med. 177, 1779–1784