Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 398-405

INDUCTION OF NITRIC OXIDE SYNTHASE IN HUMAN CHONDROCYTES

Richard M.J. Palmer+, Mark S. Hickery*, Ian G. Charles,
Salvador Moncada and Michael T. Bayliss*

Wellcome Research Laboratories, Langley Court,
Beckenham, Kent, BR3 3BS, UK

*Kennedy Institute of Rheumatology, Hammersmith, London, W6 7DW, UK

Received April 19, 1993

ABSTRACT: Incubation of human chondrocytes with interleukin-1ß, tumour necrosis factor or endotoxin induced the expression of NO synthase. The synthesis of NO induced by IL-1ß was concentration- and time- dependent, occurred after a lag period of approximately 6h and was inhibited by N^G-monomethyl-L-arginine, cycloheximide, dexamethasone and hydrocortisone, but not by indomethacin. The activity of NO synthase from activated chondrocytes was not affected by EGTA or by the calmodulin inhibitor W-13. Northern blot analysis, with a rabbit chondrocyte *inos* probe, showed a 4.4kb positively hybridising band from activated human chondrocytes. Thus, human articular chondrocytes express an inducible NO synthase from the same family as the rabbit chondrocyte and rodent macrophage enzymes. This family appears to vary in terms of in vitro Ca^{2+}-dependence and sensitivity to glucocorticoids.

The induction of nitric oxide (NO) synthesis by endotoxin (LPS) and/or cytokines, such as interleukin-1 (IL-1) and tumour neurosis factor (TNF), has been demonstrated in a wide variety of mammalian cells and tissues (1). Induction of NO synthesis was first demonstrated in rodent macrophages (2,3) a finding that has been extended to other cells including porcine vascular endothelium (4), rat hepatocytes (5) and vascular smooth muscle (6,7) and, more recently, to rabbit articular chondrocytes (8,9). The functional consequences of induction of NO synthesis in all these cells and tissues is not known, however, in macrophages it is recognised as a cytotoxic effector molecule in host defence against tumour cells (3) and parasites (10,11). Induction of NO synthase in vascular smooth muscle mediates vasodilatation (6), and in endothelial cells causes cytotoxicity (12), both of which may play a role in the pathophysiology of septic shock.

Data from cloning studies have identified three different NO synthase classes; the neuronal form (13,14) and the endothelial form (15,16), both of which are constitutively expressed, and the inducible form in the rodent macrophage cell line RAW 264.7 (17,18) and the rat smooth muscle (19). Comparison of the deduced sequences of these three classes of *nos* genes shows

+To whom correspondence should be addressed.

0006-291X/93 $4.00

an overall 50-60% similarity and high homology to cytochrome p450 reductase. To date, the DNA sequence for the human forms of NO synthase have only been reported for the genes encoding the neuronal (14) and endothelial (16) forms. The expression of inducible NO synthase(s) in human cells has been difficult to demonstrate and often occurs only in response to complex mixtures of stimuli (20,21). There is evidence, however, for increased NO synthesis in humans with sepsis (22) and in synovial fluid from arthritic patients (23).

Articular chondrocytes occupy a central position in the regulation of cartilage homeostasis, through the synthesis and degradation of matrix components (24). A number of inflammatory mediators, including IL-1 (25) and LPS (26), modulate chondrocyte function, and thus cartilage homeostasis, through the expression of cartilage-degrading enzymes (27) and by regulating cartilage synthesis (28). The role of NO in these processes is not known, but has been suggested to involve regulation of the expression of cartilage matrix-degrading enzymes through elevation of cyclic GMP (8). Since IL-1, which induces the expression of NO synthase in rabbit chondrocytes, is an important regulator of chondrocyte function, we have examined the induction of this enzyme in human chondrocytes and have partially characterised this enzyme biochemically, pharmacologically and at a molecular level.

MATERIALS AND METHODS

Materials: Penicillin, streptomycin, modified Eagle's medium, foetal calf serum, glutamine, non-essential amino acids (all Gibco); trypsin, collagenase, L-arginine, dexamethasone, W-13, cycloheximide (all Sigma); NADPH (Boehringer); human IL-1ß, TNF (British Biotechnology); N^G-monomethyl-L-arginine (Wellcome); tetrahydrobiopterin (Shirks) and LPS (W.S. typhosa: Difco) were obtained as indicated. Oligonucleotides were synthesised on a Milligen oligonucleotide synthesiser (Millipore, UK). All other chemicals and reagents were of Analar grade.

Isolation and culture of human chondrocytes: Human articular chondrocytes were isolated from human articular cartilage obtained from knees of patients undergoing amputation or massive joint replacement for osteosarcoma or chondrosarcoma distant from the joint, as described previously (29). Briefly, full thickness slices of cartilage were washed in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 2mM glutamine, non-essential amino acids and 2% antibiotic/antimycotic (DMEM). The cartilage was then finely diced prior to incubation for 45min at 37°C with pronase (700u/ml) in DMEM on a roller. The supernatant was discarded and the cartilage further digested in DMEM containing collagenase (350u/ml) for 3h as above. The supernatant was filtered through a 75µm filter and centrifuged at 300g for 8 min before being washed in Ca^{2+} - and Mg^{2+}-free 0.1M phosphate buffered saline, pH7.2. The remaining cartilage was further digested with collagenase (350u/ml) in DMEM as above, until no cartilage remained. The cells were harvested and washed as above, before being pooled with those from the first digest and resuspended finally in DMEM. Viability was determined by trypan blue exclusion and was always > 95%. The cells were finally cultured in 48 well plates or 25 cm^2 culture flasks under an atmosphere of 5% CO_2 in air for 48 h to allow them to adhere and acclimatise.

Incubation procedures: After culture for 48h as above, the cells were washed and incubated in DMEM containing 10% foetal calf serum, penicillin (100u/ml) and streptomycin (100µg/ml) and IL-1ß, TNF, LPS and/or drugs as appropriate. After incubation for 24hr at 37°C, the culture supernatant was removed and stored at -20°C until assayed for nitrite (NO_2^-) by chemiluminescence (30). In some experiments the cells were cultured in flasks

before being activated with IL-1ß for 24h. The cells were then harvested with trypsin, washed in growth medium and NO synthase prepared as described below. Induction of NO synthase was confirmed by determining NO_2^- in the culture supernatant as above.

Preparation and assay of NO synthase: NO synthase was prepared and assayed as described previously (9). Briefly, cells were harvested as above and washed in 0.1M Hepes buffer pH7.4 containing 1mM dithiothreitol, before being resuspended at 10^7 cells/ml in this buffer. After three cycles of freeze thawing, the homogenate was centrifuged at 100,000g for 30min and the supernatant used as the source of NO synthase. NO synthase activity was determined in incubations containing 200µl NO synthase preparation, 5µM human haemoglobin, 100µM NADPH and 1µM tetrahydrobiopterin in a total of volume of 500µl. Enzyme activity was initiated by addition of L-arginine (30µM) and was determined spectrophotometrically (31). The protein concentration in the homogenate was assayed colorimetrically (Bio Rad).

DNA and RNA procedures: Rabbit chondrocytes were isolated and induced to express NO synthase with LPS as described (9). mRNA was extracted from control and activated cells using a Fast-Track kit (Invitrogen). PCR was carried out on poly A+ mRNA using Taq polymerase following the manufacturer's recommended conditions (Cetus/Perkin Elmer, UK) for 30 cycles of denaturation, 96°C 35s; annealing, 55°C, 2 min and extension, 72°C, 3 min. Primers specific for the housekeeping gene ß actin were used to generate a 630bp band as a control for successful mRNA isolation: 5'-GATGGTGGGTATGGGTCAGAAGGA-3' (BB5) and 5'-GCTCATTGCCGATAGTGATGACCT-3' (BB6). The degenerate *nos* specific primers 5'-CGGGATCCGGNACNGGNATHGCNCCNTT-3' (BB3) and 5'-GCGAATTCNCCRCAN ACRTADATRTG-3' (BB4) derived from the NADPH-ribose and NADPH-adenine sites (15) of the rat brain *nos* (13) were used to generate a 350bp band by PCR. This band was identified, excised from the agarose gel, purified and cloned into EcoR1 - BamH1 digested Bluescript pBS SKII+ (Stratagene, UK) using standard conditions (32). DNA sequencing (33) of double stranded recombinant plasmids (34) was used to confirm the identify of *nos*-specific sequences. One such clone, pRINOS, was isolated for further study.

The 350bp *nos*-specific fragment was excised from pRINOS and 50ng was labelled with digoxigenin (Boehringer Mannheim). This *nos* probe was used in Northern blot experiments following separation of mRNA on formaldelyde-agarose gels (32) and transfer to nylon membranes (Amersham UK). The size of positively hybridising bands were estimated using a RNA ladder (Gibco BRL, UK).

Hybridisation was carried out overnight at 65°C and the filter washed under stringent conditions (0.1% SSC, 0.1% SDS). The signal was developed using digoxin antibody coupled to alkaline phosphatase with the chemiluminescent substrate 3-(2'-spirodamantane)-4-methoxy-4- (3"-phosphoryloxy) phenyl-1,2 dioxetane, disodium salt. Following washing, the filter was exposed to film for 30min and developed.

RESULTS

Incubation of human chondrocytes with IL-1ß (1ng/ml) caused the accumulation of NO_2^- in the culture supernatant after a lag of approximately 6h (Fig. 1). Cells incubated in the absence of IL-1ß did not generate NO_2^- over the same incubation period (24h). The synthesis of NO_2^- induced by IL-1ß was concentration - dependent and was half maximal at approximately 5pg/ml (Fig. 2). Human chondrocytes were also induced to synthesise NO_2^- by incubation for 24h in the presence of TNF or LPS (Fig. 2).

The synthesis of NO_2^- by human chondrocytes incubated with IL-1ß (1ng/ml; Fig. 3) was inhibited by L-NMMA (0.001 - 1.0mM) in a concentration - dependent manner. Production

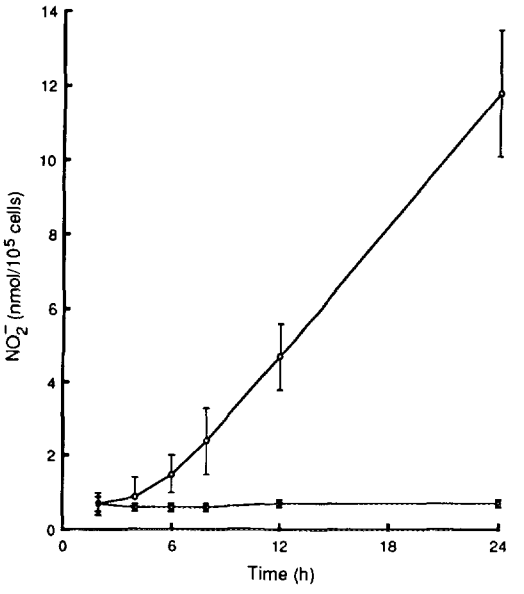

Fig. 1. Time-course of the synthesis of NO by control (■) and IL-1ß (1ng/ml;O) - stimulated human chondrocytes. Each point is the mean ± s.e.m. of three experiments.

of NO_2^- induced by IL-1β (1ng/ml) was not affected by indomethacin (5μM) whereas dexamethasone (0.1 - 1.0μM) and hydrocortisone (0.3 and 3.0μM ;n = 3 not shown) caused a small, but significant ($p<0.05$) inhibition. In contrast, cycloheximide (10μM) inhibited the synthesis of NO by approximately 75%.

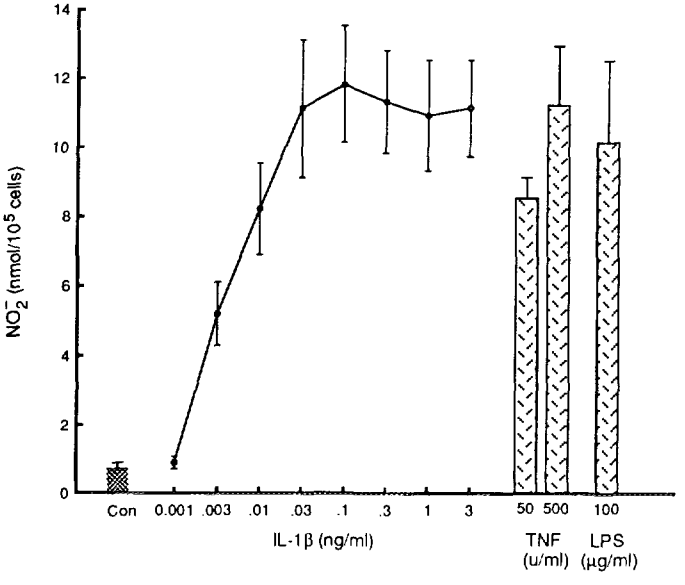

Fig. 2. Concentration - response curve for the stimulation by IL-1ß of NO synthesis after 24h. TNF (500u/ml) and LPS (100μg/ml) also induced NO synthesis after 24h. Each value is the mean ± s.e.m. of 3-4 experiments.

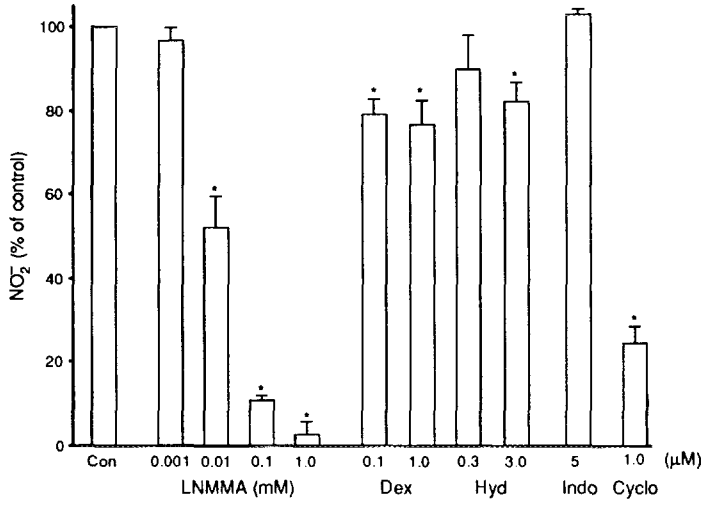

Fig. 3. Inhibition by L-NMMA, dexamethasone, hydrocortisone or cycloheximide of NO synthesis induced by IL-1ß(1ng/ml) . Indomethacin did not affect NO synthesis under these conditions (not shown). Each value is the mean ± s.e.m. of 3-4 separate experiments.

The NO synthase activity in the soluble (100,000g) fraction of homogenates of human chondrocytes incubated for 24h in the presence of IL-1ß (1ng/ml) was 216.1 ± 41.3 pmol/mg protein/min (n=3). This activity was not affected by either EGTA (1mM; n=3) or by W-13 (100μM;n=3).

The degenerate oligonucleotide primer set BB3 and BB4 were used successfully to amplify a 350bp fragment by PCR from mRNA isolated from rabbit articular chondrocytes incubated for 24h with LPS (100μg/ml). No amplified product was detected in mRNA from cells that had not been incubated with LPS. mRNA isolated from induced and noninduced rabbit cells both gave a 630bp band with the ß-actin primers BB5 and BB6, indicating that mRNA extraction was successful. DNA sequencing of the 350bp PCR product, obtained with the degenerate *nos* primers BB3 and BB4, confirmed that the fragment was highly homologous to the *inos* gene sequence published for the RAW 264.7 cell line (17,18), with only 33bp difference (data not shown).

The 350bp fragment of the rabbit chondrocyte *inos* gene obtained was used as a probe in Northern blot experiments with poly A^+ mRNA extracted from control and induced human and rabbit chondrocytes (Fig. 4). A 4.4kb positively hybridising band was present only in tracks corresponding to mRNA isolated from rabbit or human cells incubated with LPS (100 μg/ml) or IL-1ß (1ng/ml) respectively.

DISCUSSION

Human chondrocytes synthesise NO when incubated with IL-1ß, TNF or LPS. In the case of IL-1ß, this synthesis is time-and concentration-dependent, occurs after a lag period of 6h and

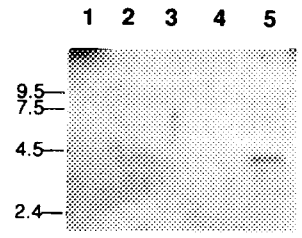

Fig. 4. Northern blot of poly A+ mRNA extracted from uninduced and induced human (tracks 1 and 2) and rabbit (tracks 4 and 5) chondrocytes; track 3 is a blank. The 350bp *nos*-specific fragment from pRINOS was used to detect a 4.4kb *nos* specific band in the induced tracks.

is inhibited by cycloheximide and L-NMMA. These data are consistent with the de novo expression of an inducible NO synthase in these cells and are similar to those previously reported for rabbit chondrocytes (9). Interestingly, human chondrocytes express NO synthase in response to a single stimulus, unlike the multiplicity of stimuli required for other human cells (20, 21). The induction of NO synthase in human chondrocytes was only partially (ca 75%) inhibited by a concentration of cycloheximide higher than that required to abolish rabbit chondrocyte NO synthase expression (9). The reason for this is not clear but it may be due to variation in the sensitivity of the 80S subunit to inhibition by cycloheximide.

The induction of NO synthase in human chondrocytes was partially (< 25%) inhibited by concentrations of dexamethasone and hydrocortisone that abolish NO synthase expression in macrophages, endothelial cells, smooth muscle and neutrophils (4, 6, 35, 36), but do not affect rabbit chondrocyte NO synthase expression. This difference may simply be a reflection of the mechanisms whereby chondrocytes, as compared to other cells, respond to IL-1. Alternatively, it may indicate differential regulation of the expression of this family of NO synthases or other factors such as species differences or age.

Northern blots using a rabbit chondrocyte *inos* specific probe showed hybridisation to a 4.4kb band mRNA extracted from activated human and rabbit chondrocytes, but not uninduced cells. This is a similar size band to that found with induced RAW 264.7 cells (17, 18). This observation, together with the high sequence identity, suggests that the rabbit and the human chondrocyte inducible enzymes belong to the same family as the macrophage *inos*. If so, then the inducible NO synthases represent a family with variable Ca2+ - dependence, probably associated with differential calmodulin affinity. Experiments are in progress to clone and sequence the full length gene for the human chondrocyte inducible NO synthase to confirm these observations.

Both IL-1 and NO_2^- are present in the synovial fluid of inflamed joints (23, 37). IL-1 affects chondrocyte function by inhibiting proteoglycan and collagen (Type II) synthesis and promoting matrix degradation by stimulating neutral metalloproteinase activity. Whether these actions are mediated, at least in part, by a NO-dependent mechanism remains to be established. The actions of NO (1) however, already suggest that it may play a role in inflammation which in addition to regulating chondrocyte function may also include effects on vasodilatation, fluid extravasation, cellular damage and modulation of leukocyte activation.

REFERENCES

1. Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991). Pharmacol. Rev. **43**, 109-141.
2. Stuehr, D.J. and Marletta, M.A. (1987). J. Immunol. **139,** 519-525.
3. Hibbs, J.B.Jr., Taintor, R.R. and Vavrin, Z. (1987). J. Immunol. **138,** 550-565.
4. Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1990). Proc. Natl. Acad. Sci. USA. **87**, 10043-10047.
5. Billiar, T.R. Curran, R.D., Stuehr, D.J., Stadler, J., Simmons, R.L. and Murray, S.A. (1990). Biochem. Biophys. Res. Commun. **168,** 1034-1040.
6. Rees, D.D., Cellek, S., Palmer, R.M.J. and Moncada, S. (1990). Biochem. Biophys. Res. Commun. **173**, 541-547.
7. Busse, R. and Mulsch, A. (1990). FEBS Lett. **265**, 133-136.
8. Stadler, J., Stefanovic-Racic, M., Billiar, T.R., Curran, R.D., McIntyre, L.A., Georgescu, H.I., Simmons, R.L. and Evans, C.H. (1991). J. Immunol. **147**, 3915-3920.
9. Palmer, R.M.J., Andrews, T., Foxwell, N.A. and Moncada, S. (1992). Biochem. Biophys. Res. Commun. **188**, 209-215.
10. James, S.L. and Hibbs, J.B.Jr., (1991). Parasitology Today, 6, 303-305.
11. Liew, F.Y., Millot, S., Parkinson, C., Palmer, R.M.J. and Moncada, S. (1990). J. Immunol. **144**, 4794-4797.
12. Palmer, R.M.J., Bridge, L. Foxwell, N.A. and Moncada, S. (1992). Br. J.Pharmac. **105**, 11-12.
13. Bredt, D.S., Hwang, P.H., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H. (1991). Nature, **351**, 714-718.
14. Nakane, M., Schmidt, H.H.H.W., Pollock, J.S., Forstermann, U. and Murad, F. (1993). FEBS Lett. **316**, 175-180.
15. Janssens, S.P., Shimouchi, A., Quertermous, T., Bloch, D.B. and Bloch, K.D.(1991). J. Biol. Chem. **267**, 14519-14522.
16. Marsden, P.A., Schappert, K.T., Chen, H.S., Flowers, M., Sundell, C.L., Wilcox, J.N., Lamas, S. and Michel, T. (1992). FEBS Lett. **307**, 287-293.
17. Lyons, C.R. Orloff, G.J. and Cunningham, J.M. (1992). J. Biol. Chem. **267**, 6370-6374.
18. Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. and Nathan, C., (1992). Science, **256**, 225-228.
19. Nunokawa, Y., Ishida, N. and Tanaka, S. (1993). Biochem. Biophys. Res. Commun. **191**, 89-94.
20. Nussler, A.K., Di Silvio, M., Billiar, T.R., Hoffman, R.A., Geller, D.A., Selby, R., Madriaga, J. and Simmons, R.L. (1992). J. Exp. Med. **176**, 261-264.
21. Denis, M. (1991). J.Leuk. Biol. **49**, 380-387.
22. Ochoa, J.B., Udekwu, A.O., Billiar, T.R., Curran, R.D., Cerra, F.B., Simmons, R.L.and Pietzman, A.B. (1991). Ann. Surg. **214**, 621-626.
23. Farrell, A.J., Blake D.R., Palmer, R.M.J. and Moncada, S. (1992). Ann. Rheum. Dis. **51,** 1219-1222.
24. Morales, T.I. and Hascall, V.C. (1989). Arth. Rheum. **32,** 1197-1201.
25. Tyler, J.A., (1985). Biochem. J. **227**, 869-878.
26. Morales, T.I. and Hascall, V.C. (1989). Connect. Tiss. Res. **19**, 255-275.
27. Murphy, G. and Reynolds, J.J. (1985). Bio Essays **2**, 55-60.
28. Benton, H.P. and Tyler, J.A. (1988). Biochem. Biophys. Res. Comm. **154**, 421-428.
29. Archer, C.W., McDowell, J., Bayliss, M.T., Stephens, M.D. and Bentley, G. (1990). J. Cell. Sci. **97**, 361-371.
30. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nature (Lond.) **327**, 524-526.
31. Feelisch, M. and Noack, E. (1987). Eur. J. Pharmac. **139**, 19-30.

32. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. Sanger, F., Nichlen, S. and Coulson, A.R. (1983). Proc. Natl. Acad. Sci. USA **74**, 5463-5467.
34. Stephen, D., Jones, C. and Schofield, J.P. (1991). Nucleic Acids Res. **24**, 7463-7464.
35. Di Rosa, M., Radomski, M.W., Carnuccio, R. and Moncada, S. (1990). Biochem. Biophys. Res. Commun. **172**, 1246-1252.
36. McCall, T.B., Palmer, R.M.J. and Moncada, S. (1991). Eur. J. Immunol. **21**, 2523-2527.
37. Arend, W.P. and Dayer, J.M. (1990). Arthritis. Rheum. **33**, 305-315.