Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 209-215

GLUCOCORTICOIDS DO NOT AFFECT THE INDUCTION OF A NOVEL CALCIUM-DEPENDENT NITRIC OXIDE SYNTHASE IN RABBIT CHONDROCYTES

Richard M.J. Palmer*, Tara Andrews, Neale A. Foxwell and Salvador Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, U.K.

Received August 13, 1992

ABSTRACT: Incubation of rabbit articular chondrocytes with interleukin-1β caused time-dependent expression of NO synthase, determined as nitrite, after a lag period of 6h. The synthesis of nitrite was concentration-dependent and was inhibited by cycloheximide and N^G-monomethyl-L-arginine, but not by dexamethasone or hydrocortisone. The synthesis of NO in the 100,000g supernatant of activated chondrocytes was inhibited by EGTA, but not by the calmodulin inhibitors W-13 or trifluoperazine. The synthesis of NO was half-maximal at approximately 20nM free Ca^{2+}. Endotoxin also induced the expression of this NO synthase. Thus, rabbit articular chondrocytes express a novel inducible NO synthase which is Ca^{2+}-dependent, and whose induction is not prevented by glucocorticoids.

The synthesis of nitric oxide (NO) from the amino acid L-arginine has recently been shown to be a widespread mechanism for regulating cell function and communication (1). At present, two general classes of NO synthase are recognised; one is Ca^{2+}/ calmodulin-dependent and is present constitutively in a number of cells including the vascular endothelium, central and peripheral nerves and platelets (2). The other is Ca^{2+}-independent and is synthesised de novo in cells, such as macrophages and vascular smooth muscle, following exposure to endotoxin (LPS) and/or cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF). The expression of the latter class of NO synthase is inhibited by glucocorticoids (3). A number of isoforms of these general classes of this enzyme have been described, which differ as to whether they are soluble or particulate and constitutive or inducible (4).

The constitutive enzyme synthesises NO as a transduction mechanism for the activation of soluble gunaylate cyclase (2), whilst the inducible enzyme synthesises NO

*To whom correspondence should be addressed.

0006-291X/92 $4.00

as a cytotoxic molecule contributing to host defence (5). Inappropriate expression of the inducible enzyme also plays a role in some diseases such as septic shock, where increased NO synthesis in the vessel wall leads to excessive vasodilatation (6) and endothelial damage (7).

Articular chondrocytes are able both to synthesise and degrade proteoglycans and therefore occupy a central position in the homoeostatic regulation of cartilage (8). The balance between these two processes is modulated by a number of agents that contribute to inflammation, including IL-1 (9) and LPS (10). The production of IL-1 by a variety of cells in the inflamed joint inhibits proteoglycan synthesis (11) and induces the expression of cartilage-degrading enzymes (12) by chondrocytes, leading to degradation of cartilage matrix.

Since IL-1 and LPS induce the expression of the Ca^{2+}-independent inducible NO synthase in a number of cells and tissues (2), we have examined whether rabbit articular chondrocytes express NO synthase when stimulated with IL-1 or LPS. Furthermore, we have partially characterised the enzyme in these cells and have found that it differs signficantly from the inducible enzyme previously characterised in other cells. Recently, Stadler et al (13) have independently shown that rabbit articular chondrocytes stimulated with IL-1, TNF and LPS synthesis NO and suppress gelatinase and prostaglandin E_2 synthesis.

MATERIALS AND METHODS

Materials: Penicillin, streptomycin, modified Eagle's medium (MEM), foetal calf serum, glutamine (all Gibco); trypsin, collagenase, hyaluronidase, L-arginine, dexamethasone, hydrocortisone, W-13, trifluoperazine, L-nitro-arginine methyl ester, cycloheximide (all Sigma); dispase, NADPH (Boehringer); human IL-1β, TNF (British Biotechnology); N^G-monomethyl-L-arginine (Wellcome); tetrahydrobiopterin (Shirks) and LPS (W.S. Typhosa; Difco) were obtained as indicated and all other chemicals and reagents were of Analar grade.

Isolation and culture of rabbit articular chondrocytes: Chondrocytes were isolated and cultured as described previously, with minor modifications (14). Male New Zealand White rabbits (2.5kg) were given an overdose of sodium pentobarbitone and the articular surface of the knee exposed. Cartilage was removed, washed and chopped into small pieces (approx $2mm^3$). The cartilage was then digested with hyaluronidase (1mg/ml) for 15 min followed by trypsin (2.5mg/ml) for 30 min, dispase (5mg/ml) for 18h and collagenase (2mg/ml) for 6h. The resulting chondrocyte suspension was washed and the cells cultured in MEM containing penicillin (100U/ml), streptomycin (100µg/ml), glutamine (2mM) and foetal calf serum (20% v/v). Chondrocytes passaged between two and four times were used in all experiments.

Incubation procedures: Cells were subcultured into 24-well plates until confluent. The growth medium was then replaced with 1ml fresh medium and IL-1β, LPS, TNF and/or drugs as appropriate. After incubation at 37°C for up to 48h (generally 24h), the culture supernatant was removed and stored at -20°C until assayed for nitrite (NO_2^-) by chemiluminescence (15). In some experiments the cells were cultured in flasks before being activated with IL-1β (1ng/ml) for 24h. The cells were then harvested with trypsin, washed in growth medium and NO synthase prepared as described below. Activation of the cells was confirmed by determining NO_2^- in the culture supernatant.

Preparation and assay of NO synthase: The cells were washed in 0.1M Hepes buffer pH7.4, containing dithiothreitol (1mM) and were resuspended in this buffer at 10^7 cells/ml. After three cycles of freeze-thawing, the homogenate was centrifuged at 100,000g for 30 min and the supernatant containing the soluble fraction used as the source of NO synthase. Enzyme activity in incubations, containing 200µl NO synthase preparation, 5µM human haemoglobin, 100µM NADPH, 5µM tetrahydrobiopterin and 30µM L-arginine in a total volume of 500µl, was assayed spectrophotometrically as described (16). The protein concentration in the homogenate was determined colorimetrically (Bio Rad).

RESULTS

There was no significant rise in NO_2^- over 48h in the culture supernatant of chondrocytes incubated in medium alone (Fig. 1). In contrast, the amount of NO_2^- in the supernatants of cells incubated in the presence of IL-1β (3ng/ml) increased linearly throughout the incubation period, following a lag of approximately 6h (Fig. 1).

The increase in NO_2^- synthesis induced by IL-1β at 24h was concentration-dependent (Fig. 2) and was half maximal at approximately 20pg/ml. Incubation with LPS (1-100µg/ml) for 24h also increased the amount of NO_2^- in a concentration-dependent manner. Thus, the amount of NO_2^- after 24h was increased to 2.8±1.6, 11.6±5.3 and 22.9±7.4 (n=3 for each) nmol/10^5 cells by 1,10 and 100µg/ml LPS respectively.

The synthesis of NO_2^- by chondrocytes incubated for 24h with IL-1β (3ng/ml; Fig. 3) was inhibited by concurrent incubation with cycloheximide (1 and 10µM) and with N^G-monomethyl-L-arginine (L-NMMA; 0.01-1.0mM). Dexamethasone (0.1 and 1µM), hydrocortisone (0.3 and 3.0µM) and indomethacin (5µM) had no significant effect on NO_2^- synthesis under these conditions (Table 1). L-NMMA also caused similar inhibition of NO_2^- synthesis (Table 1) induced by a submaximal concentration of IL-1β

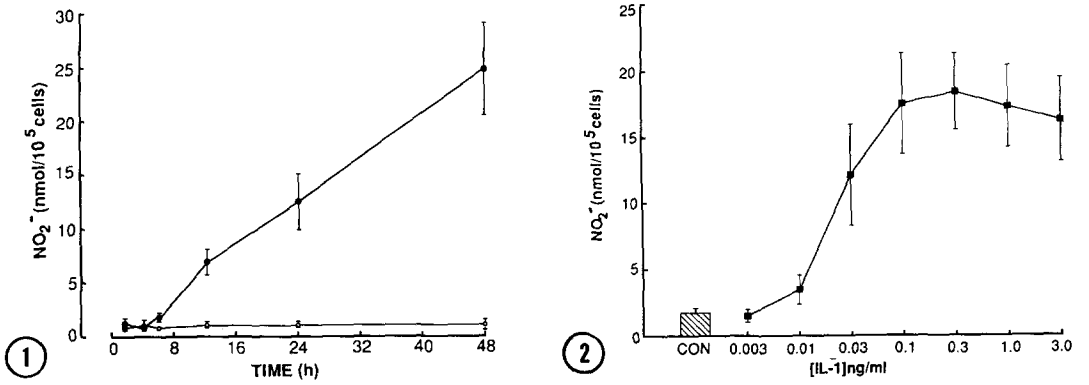

Fig.1. Time course of the synthesis of NO_2^- by control (o) and IL-1β (3ng/ml; ●) stimulated chondrocytes. Each point is the mean±s.e.m. of four experiments.

Fig.2. Concentration-response curve for the stimulation by IL-1β of NO_2^- synthesis by chondrocytes incubated for 24h. Each point is the mean±s.e.m. of three experiments.

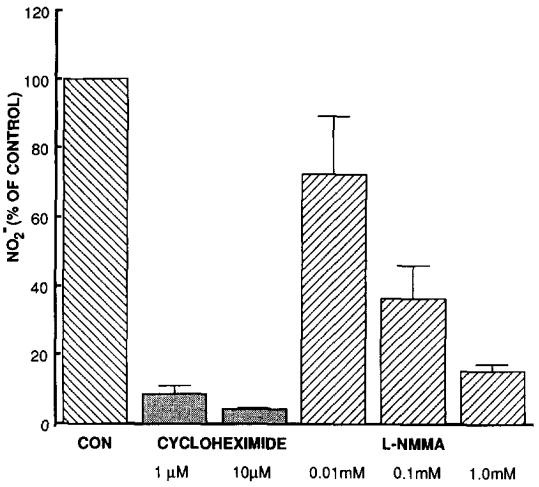

Fig.3. Effect of cycloheximide and L-NMMA on the synthesis of NO_2^- by chondrocytes stimulated with IL-1β (3ng/ml) for 24h. NO_2^- synthesis was inhibited by both cycloheximide and by L-NMMA. Each point is the mean±s.e.m. of three experiments.

(0.03ng/ml) and by LPS (100μg/ml). Culture of the cells in L-arginine (400μM)-containing medium, followed by 24h in L-arginine-free medium containing IL-1β (3ng/ml), reduced the synthesis of NO_2^- by 66.7±1.2% (n=3).

Table 1. Effects of various compounds on the synthesis of NO_2^- by chondrocytes

	NO_2^- synthesis (% of control)		
	IL-1 3.0 ng/ml	IL-1 0.03 ng/ml	LPS 100μg/ml
Dexamethasone			
1.0μM	81.5±5.2	81.9±7.1	93.1±7.8
0.1μM	97.0±14.3	91.6±3.4	94.7±3.7
Hydrocortisone			
3.0μM	92.9±9.7	94.8±8.0	91.8±4.0
0.3μM	101.1±14.2	N.D.	90.6±4.7
Indomethacin			
5.0μM	100.1±4.2	N.D.	N.D.
L-NMMA			
1.0mM	15.3±1.9	17.8±2.1	11.2±0.9
0.1mM	36.1±9.6	38.0±5.2	40.9±1.0
0.01mM	72.3±16.8	88.1±8.4	82.8±2.7

N.D. = not done.

Cells incubated for 24h with IL-1β or LPS. Each value is the mean±s.e.m. of three or four experiments.

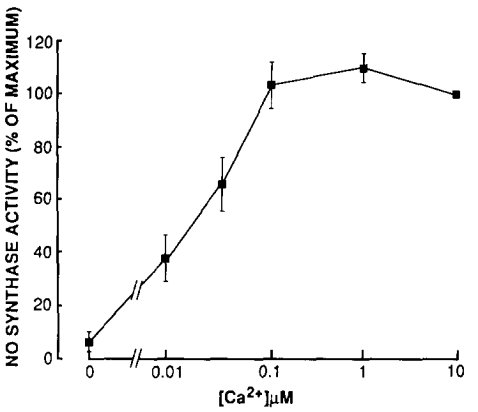

Fig. 4. Ca^{2+}-dependence of the inducible NO synthase from chondrocytes stimulated with IL-1β (1ng/ml) for 24h. NO synthase activity was almost abolished by EGTA (1mM; zero free Ca^{2+}) and was restored in a concentration-dependent manner by increasing the free Ca^{2+} concentration. Each point is the mean±s.e.m. of three experiments.

The NO synthase activity in the soluble (100,000g) fraction of homogenates of chondrocytes activated for 24h in the presence of IL-1β (1ng/ml) was inhibited by EGTA (1mM; Fig. 4), but not by the calmodulin inhibitors W-13 or trifluoperazine (100µM; n=3 for each). Experiments with Ca^{2+}/EGTA buffers showed that NO synthase activity was half maximal at a concentration of approximately 20nM free Ca^{2+} (Figure 4).

DISCUSSION

Stimulation of rabbit articular chondrocytes with IL-1β causes concentration-and time-dependent synthesis of NO_2^-, which is inhibited by L-NMMA, an inhibitor of the synthesis of NO from L-arginine by both constitutive and inducible NO synthases (1,2). The synthesis of NO_2^- occurs after a lag period of approximately 6h and is inhibited by cycloheximide, indicating that it is due to the de novo expression of an inducible NO synthase. A similar induction of NO synthesis in rabbit cartilage explants stimulated with IL-1β has also been observed (our unpublished results).

The expression of NO synthase in chondrocytes is not affected by dexamethasone or hydrocortisone, at concentrations that abolish the induction by LPS of the Ca^{2+}-independent inducible enzyme in macrophages (17), vascular endothelial cells (18) and vascular smooth muscle (6). The lack of effect of these compounds cannot be attributed to the use of a maximum effective concentration of IL-1β, since they also had no effect on the induction of the NO synthase by an approximately half maximally effective concentration of IL-1β. Furthermore, it is unlikely to be due to different intracellular mechanisms for IL-1β compared to LPS, as the induction by LPS of NO synthase in

chondrocytes was also not affected by these compounds. This finding suggests that the expression of the NO synthase in chondrocytes may be regulated differently from that for the Ca^{2+}-independent enzyme in other cells.

The expression of NO synthase is not affected by indomethacin. In other cells, such as macrophages (19) and vascular endothelial cells (7) there is evidence to suggest that prostanoids down-regulate the synthesis of NO by the Ca^{2+}-independent enzyme. This does not appear to be the case in chondrocytes, which synthesise PGE_2 when stimulated with IL-1β (20). This provides further support for the proposal that this gene is regulated differently. This finding also suggests that, if the synthesis of NO by IL-1β-stimulated chondrocytes plays a role in cartilage homoeostasis, then it exerts its effect independently from that of PGE_2.

The NO synthase induced in chondrocytes by IL-1β is soluble, Ca^{2+}-dependent and appears to be calmodulin-independent, although it is possible that calmodulin may be tightly bound to the enzyme. These properties distinguish it from the constitutive enzyme in endothelial cells and brain (2) and from the Ca^{2+}-dependent constitutive enzyme described in human neutrophils (21). This finding, together with the lack of effect of glucocorticoids, supports the proposal that the inducible NO synthase in rabbit chondrocytes represents a novel class of this family of enzymes. The Ca^{2+}-dependent NO synthase activity observed in the rat ileum following treatment for 6h in vivo with LPS (22) may also belong to this class of NO synthase.

The significance of the induction of NO synthase in inflammation and homoeostasis of the joint is not known. IL-1β inhibits proteoglycan synthesis and induces the expression of cartilage matrix-degrading enzymes (11-13), and IL-1β concentrations increase in the inflamed joint (23). However, the precise mechanisms that regulate cartilage destruction are not clearly established. Nitric oxide is able to affect cell function either by activating soluble guanylate cyclase to elevate cGMP (2) or by interaction with iron-containing enzymes, such as those involved in respiration and cell division (5), both of which actions could underly the actions of NO as an inflammatory mediator in the joint.

REFERENCES

1. Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1989). Biochem. Pharm. **38**, 1709-1715.
2. Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991). Pharmacol. Rev., **43**, 109-141.
3. Moncada, S. and Palmer, R.M.J. (1991). Trends in Pharmacol. Sci., **12**, 130-131.
4. Forstermann, U., Schmidt, H.H.H.W., Pollock, J.S., Sheng, H., Mitchell, J.A., Warner, T.D., Nakane, M. and Murad, F. (1991). Biochem. Pharmacol. **42**, 1849-1857.
5. Hibbs, J.B. Jr., Taintor, R.R., Vavrin, Z., Granger, D.L., Drapier, J-C., Amber, I.J. and Lancaster, J.R. (1990). In : Nitric oxide from L-arginine: A bioregulatory system. Ed. Moncada, S. and Higgs E.A., 189-223, Elsevier, Amsterdam.

6. Rees, D.D., Cellek, S., Palmer, R.M.J. and Moncada, S. (1990). Biochem. Biophys. Res. Commun. **173**, 541-547.
7. Palmer, R.M.J., Bridge, L., Foxwell, N.A. and Moncada, S. (1992). Br. J. Pharmac. **105**, 11-12.
8. Morales, T.I. and Hascall, V.C. (1989). Arth. Rheum. **32**, 1197-1201.
9. Tyler, J.A. (1985). Biochem. J. **227**, 869-878.
10. Morales, T.I. and Hascall, V.C. (1989). Connect. Tissue Res. **19**, 255-275.
11. Benton, H.P. and Tyler, J.A. (1988). Biochem. Biophys. Res. Commun. **154**, 421-428.
12. Murphy G. and Reynolds J.J. (1985). BioEssays **2**, 55-60.
13. 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.
14. Treschel, W., Dew, G., Murphy, G. and Reynolds, J.J. (1982). Biochem. Biophys. Acta. **720**, 364-370.
15. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nature (Lond) **327**, 524-526.
16. Feelisch, M. and Noack, E. (1987). Eur. J. Pharmac. **139**, 19-30.
17. Di Rosa, M., Radomski, M., Carnuccio, R. and Moncada, S. (1990). Biochem. Biophys. Res. Commun. **172**, 1246-1252.
18. Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1990). Proc. Natl. Acad. Sci. USA. **87**, 10043-10047.
19. Mulsch, A., Hauschildt, S., Bessler, W.G., Bassenge, D. and Busse, R. (1990). In: Nitric oxide from L-arginine: A bioregulatory system. Ed. Moncada, S. and Higgs, E.A. 235-242. Elsevier, Amsterdam.
20. Dayer, J.M. (1985). Br. J. Rheumatol. **24** Suppl. **1**, 15-20.
21. Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Ohkawa, S., Ohnishi, K., Terao, S. and Kawai, C. (1991). J. Biol. Chem. **266**, 3369-3371.
22. Salter, M., Knowles, R.G. and Moncada, S. (1991). FEBS Lett., **291**, 145-149.
23. Arend, W.P. and Dayer, J.M. (1990). Arthritis. Rheum., **33**, 305-315.