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CULTURED OSTEOBLAST SYNTHESIZE NITRIC OXIDE IN RESPONSE TO CYTOKINES AND LIPOPOLYSACCHARIDE

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INDEXING WORDS

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SYNOPSIS

Nitric oxide (NO) is generated from L-arginine by NO synthase. NO has been reported to be produced by a variety of cell types such as vascular endothelial cells, macrophages, neutrophils and articular chondrocytes. A recent report demonstrated that NO inhibits osteoclast (OC) function and, in this way, is critically associated with bone metabolism. In the present study we have studied NO synthesis by osteoblasts (OBs). OB cell line, MC3T3-E1, was cultured with the various cytokines for 72 hrs. Nitrite, a stable endproduct of cell-generated NO, in the culture supernatant was then determined by using a spectrophotometric method based on Griess reaction. IL-1α increased nitrite release in a dose-dependent fashion and a significant enhancement ($p<0.01$) was attained at 10 U/ml. OBs released 14.2 nmol/4.0X10^4 cells of nitrite after 72 hrs stimulation by 100U/ml IL-1α. In contrast IL-1β, TNF-α and INF-γ failed to affect NO synthesis by MC3T3-E1. The results suggest that OBs produce NO in response to IL-1α and OB-induced NO may play a role in OB-OC interaction in the inflammatory

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process.

INTRODUCTION

Nitric oxide (NO) has many of the biological properties such as relaxation of vascular smooth muscle and inhibition of platelet adhesion and aggregation. It has been demonstrated that not only vascular endothelial cells[14,15] but also many other cells produce NO[1,2,7,9,11,12,13,16,18] and that the typical stimuli to induce the synthesis of NO are lipopolysaccharide (LPS) and various cytokines. On the other hand, cytokines may have a role in the modulation of bone metabolism, affecting bone formation or resorption.[5] Furthermore a recent study showed that NO has an inhibitory effect on osteoclast function[10] and, in this way, is critically associated with bone metabolism. In order to investigate whether osteoblast have the capability of the NO production, experiments were undertaken to determine whether cytokines induce the synthesis of NO by osteoblasts. Also the effects of glucocorticoids, which may have a role on bone metabolism, the NO generation by OB were investigated.

MATERIALS AND METHODS

MC3T3-E1 culture

The MC3T3-E1 cell line, derived from newborn C57BL/6 mouse calvaria was provided by Dr. H. Kodama.[19] The cells were seeded into 96-well plates (Corning, Cambridge, MA., U.S.A.) at $2.0X10^5$ cells/ml in 200 μl of alpha modification of Eagle's medium (Flow Laboratories, North Ryde, NSW., Australia) containing 10 % fetal bovine serum (MA Bioproduct Walkesville, MD., U.S.A.) respectively. After the incubation for 12 hrs, the cells were replaced in fresh medium supplemented with 1% fetal bovine serum and containing various cytokines (IL1α, IL-1β, IFN-γ, TNF-α, Genzyme Corporation, Cambridge, MA., U.S.A.) and LPS (SIGMA, St. Louis, MO., U.S.A.). In order to investigate the effect of the L-arginine, ENDOTHELIAL SFM medium (GIBCO, Grand Island, NY., U.S.A.), which is free of L-arginine was used and each concentration of L-arginine was

made. Also the effect of N-monomethyl-L-arginine (NMA; SIGMA), a competitive inhibitor of L-arginine dependent NO production,[8] or glucocorticoids (prednisolone, hydrocortisone, dexamethasone) on induction of NO generation were investigated. All culture incubations were carried out at 37 C° and 5 % CO_2 and culture media were supplemented with 10^5 U/liter penicillin.

The determination of cell generated NO

As a stable endproduct of cell generated NO, NO2 (Nitrite) was determined using a spectrophotometric method based on the Griess reaction.[6] Briefly, 100 µl of the culture supernatant was incubated with an equal volume of Griess reagent (1 % sulfanilamide / 0.1 % naphthylene diamine dihydrochloride / 2.5 % H_3PO_4) at room temperature for 10 min in the 96-well microtiter plate. The absorbance was measured at 550 nm in a microplate reader and nitrite concentration was determined using a curve calibrated from sodium nitrite standards.

Assay of DNA synthesis

In order to investigate the effect of cytokines and LPS on MC3T3-E1 cell proliferation, we examined DNA synthesis of MC3T3-E1 cells. The cells were seeded into 96-well plates at $2.0X10^4$ cells/ml in 200 µl of alpha modification of Eagle's medium containing 10 % fetal bovine serum. After an appropriate period of treatment with TNF-α and LPS, the cells were labeled for 24 hrs with 1 µCi of ^{3}H-thymidine (^{3}H-TdR). Then the OBs were washed three times with warm phosphate-buffered saline and detached by trypsinization. The OBs were harvested onto glass fiber paper, using an automatic harvester and counted in a liquid scintillation counter.

Statistical analysis

Data are represented as mean ± SD. Where indicated statistical significance of differences of means was determined using the Student's t-test for unpaired groups of data.

RESULTS

Induction of NO biosynthesis by cytokines and LPS.

In order to determine whether NO production is inducible in clone MC3T3-E1 cells, the cells were exposed to various stimuli for 72 hrs. Mediators were chosen either for their capacity to induce NO biosynthesis in other cell types or well known activators of bone metabolism. NO generation was profoundly stimulated by IL-1α or LPS. In contrast, IL-1β, TNF-α or INF-γ failed to affect NO synthesis by MC3T3-E1(Table I). IL-1α or LPS increased nitrite release in a dose-dependent fashion with a significant enhancement ($p<0.01$) attained at 10 U/ml or 10 μg/ml, respectively (Fig. 1 a, b).

Cytokines and LPS synergize in induction of NO biosynthesis.

Various combinations of stimulator agents were tested for additive or synergistic effects of NO production. The combination of LPS and TNF-α resulted in a synergistic increase of NO synthesis (Fig.1 b). However, no significant elevation of NO production was observed when LPS was combined with IL-1α, β, or INF-γ (data not shown). Eventhough, TNF-α alone did not stimulate NO production, TNF-α (10 U/ml) significantly enhanced NO production in combination with IL-1α or LPS, (Fig.1 a, c). In contrast, the combination of IL-1α and LPS did not result in a synergistic increase of NO synthesis (Fig.1 a).

Time kinetics of NO release.

After the addition of IL-1α, measurable NO biosynthesis started after 6 hrs. Nitrite release was increased in a time-dependent fashion and 19 nmol/2.0×10^5 cells of nitrite was observed after 96 hrs incubation (Fig.2).

Dose-response of L-arginine and the effect of NMA.

TABLE I. Induction of NO generation in response to various cytokines and LPS.

Stimulatory Agents	Concentrations	NO_2^-(nmol/2.0x10^{5}cells/72h) *
Nil	——	1.4 ± 0.6
IFN-γ	100 U/ml	1.2 ± 0.9
IL1-α	100 U/ml	11.3 ± 0.7 **
IL1-β	100 U/ml	1.7 ± 1.0
TNF-α	500 U/ml	1.1 ± 0.5
LPS	10 μg/ml	3.7 ± 1.0 **

Osteoblasts culture were stimulated with various cytokines and LPS and incubated for 72 hours .
* Values represent the mean ± S D.
** p<0.01 v.s. without agents.

To study the L-arginine dependent NO biosynthesis, MC3T3-E1 cells were incubated with increasing concentrations of L-arginine and stimulated with 100 U/ml IL-1α. In the absence of L-arginine, no production of NO was observed. And dose-dependent nitrite release was observed up to 0.5 mM L-arginine (Fig.3). Furthermore, when the cells were incubated in the presence of 1.0 mM NMA, competitive inhibitor of L-arginine, no NO synthesis was detected (Table II).

Inhibition of NO production by glucocorticoids.

To investigate the effect of glucocorticoids, which may have an important role on the bone metabolism, MC3T3-E1 cells were incubated with dexamethasone, prednisolone, and hydrocortisone in the presence of 50 U/ml IL-1α. The significant suppression of NO generation was observed by dexamethasone at $1.0x10^{-8}$ M and about 75 % of the NO production was suppressed at $1.0x10^{-7}$ M (Fig. 4 a). Prednisolone also suppressed 70 % of the NO production at the concentration of $1.0x10^{-7}$ M (Fig. 4 b) and hydrocortisone have the inhibitory effect on NO synthesis in a similar manner (data not shown).

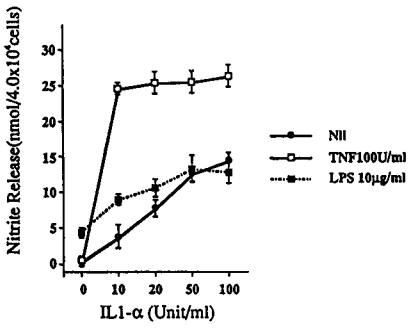

Fig. 1 a

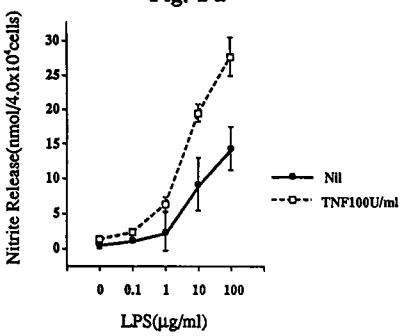

Fig. 1 b

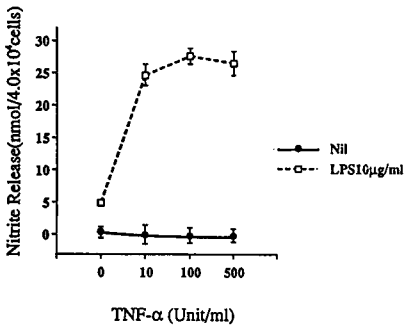

Fig. 1 c

Fig. 1. Induction of NO generation in response to cytokines and LPS.

MC3T3-E1 cells were stimulated for 72 hours with various concentrations of IL-1α (a), LPS (b), TNF-α (c) as indicated. Points and bars represent the mean ± S D.

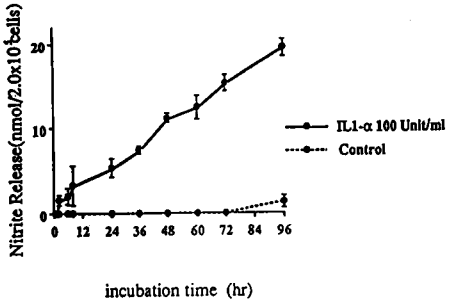

Fig. 2. Time kinetics of NO production by IL-1α.

Osteoblasts were stimulated with 100 units / ml IL-1α for various periods of time as indicated. Points and bars represent the mean ± S D.

TABLE II. Competitive inhibition of L-arginine dependent NO production by NMA.

	NO_2^-(nmol/2.0x10⁵cells/72h) *	
	NMA(-)	NMA 1mM
control	1.9 ± 0.6	2.3 ± 0.7
IL1 α (100 U/ml)	9.6 ± 1.5	2.1 ± 0.5 **

Osteoblasts were stimulated with 100 units / ml IL-1α and incubated with or without NMA for 72 hours.
* Values represent the mean ± S D.
** $p<0.01$ v.s. without NMA.

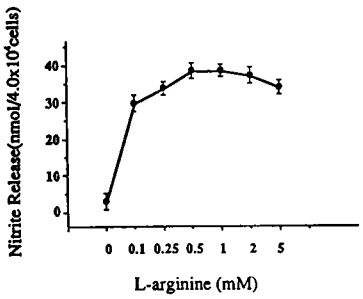

Fig. 3. The effect of L-arginine on NO production by IL-1α.

Osteoblasts were stimulated with 100 units / ml IL-1α and incubated with various concentrations of L-arginine as indicated for 72 hours. Points and bars represent the mean ± S D.

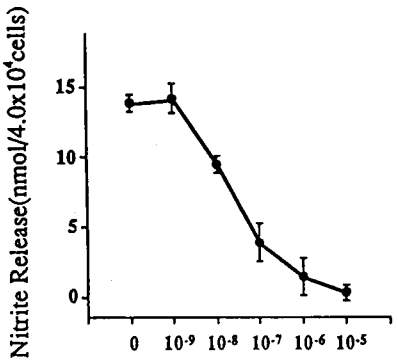

Fig. 4 a

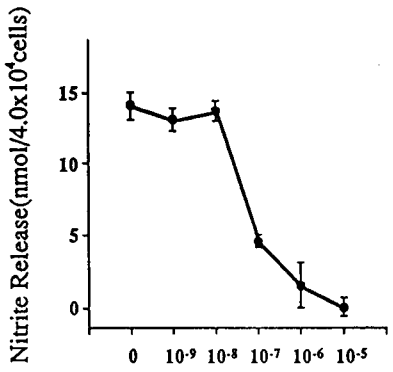

Fig. 4 b

Fig. 4. **The effect of glucocorticoids in the NO generation.**

Osteoblasts were stimulated with 50 units / ml IL-1α and incubated with increasing concentrations of dexamethasone (a) and prednisolone (b) for 72 hours. Points and bars represent the mean ± S D.

DNA synthesis.

As described previously, the combination of LPS and TNF-α resulted in a synergistic increase of NO synthesis. We next investigated the effect of this combination on the DNA synthesis in MC3T3-E1 cells. MC3T3-E1 cells were incubated with increasing concentrations of TNF-α in the presence or absence of 10 μg/ml LPS. Also the cells were incubated with increasing concentrations of LPS in the presence or absence of 100 U/ml TNF-α. TNF-α did not affect on the DNA synthesis in MC3T3-E1 cells. Eventhough, the DNA synthesis in MC3T3-E1 cells were slightly suppressed, the inhibitory effect was not statistically significant. The combination of LPS and TNF-α also had no significant effect (Fig. 5). These results suggest that enhanced NO production induced by TNF-α and LPS does not depend on the effect of cell proliferation. Moreover, the combination of IL-1α and TNF-α had no significant effect on DNA synthesis (data not shown).

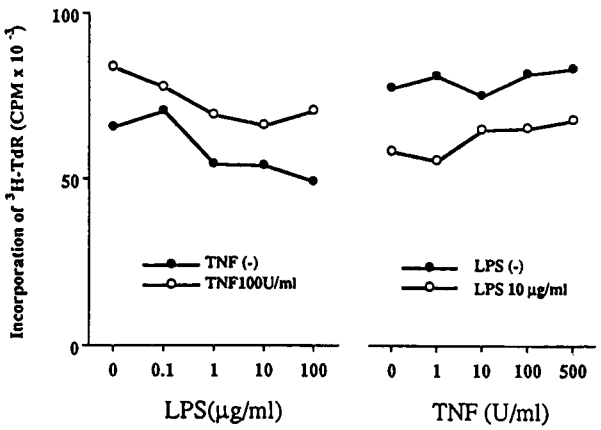

Fig. 5. **The effect of the combination of LPS and TNF-α on the DNA synthesis in MC3T3-E1 cells.**

Osteoblasts were incubated under various concentrations of LPS or TNF-α with or without the other agent for 48 hrs. Then the cells were labeled with 3H-TdR for 24h.

DISCUSSION

NO plays an important role in intercellular communication of neutrophils, brain tissue, renal epithelial cells, mast cells, and autonomic nerves, in addition to its known function as a vasodilator produced by the vascular endothelial cells.[20] Furthermore, it was recently demonstrated that NO also has a strong inhibitory effect on the bone resorbing activity of the osteoclast.[10] In the present study, we demonstrated that NO biosynthesis could be inducible in osteoblast cell line, MC3T3-E1 cells by IL-1α and LPS, though, no spontaneous production of NO was observes. Dose-dependent nitrite release by L-arginine was observed and NMA, a competitive inhibitor of L-arginine dependent NO production, blocked NO production. These facts suggest that nitric oxide synthase (NOS) could be induced by IL-1α and LPS. Similar to other cell types containing an inducible NOS, detectable NO production in clone MC3T3-E1 cells was started after 6 hrs and continued thereafter. In macrophages[4] and hepatocytes,[3] IL-1 dose not induce NO production by itself. IFN-γ plays a major role in the induction of NO biosynthesis in most type of cells.[21] However, IFN-γ is capable of inducing NO generation neither in articular chondrocytes[17] nor in MC3T3-E1 cells. IL-1α is very important in the induction of NO biosynthesis both in the osteoblasts and the articular chondrocytes. Therefore osteoblasts are very similar to articular chondrocytes but differ from other cell types with respect to the mediators capable of inducing NO generation.

Synergistic enhancement of NO production was observed when the cells were stimulated by TNF-α in combination with LPS or IL-1α. In contrast, the combinations of TNF-α and LPS or TNF-α and IL-1α have not affected DNA synthesis in MC3T3-E1 cells. Moreover TNF-α alone could not induce NO production in MC3T3-E1 cells. It is well known that IL-1 is one of the strongest local regulatory factor of bone resorption.[5] However NO, which may have a inhibitory effect on the bone-resorbing activity of the osteoclast, could be generated by IL-1. The synergistic induction of NO by osteoblast was observed when the cells were stimulated by TNF-α in combination with IL-1α. Thus the abundant NO induced by the combination

of the cytokines such as TNF-α and IL-1α might act directly on the osteoclasts and inhibit its function of bone resorption. In this way, cytokines and LPS may have the possibility to participate the feedback mechanism towards bone resorption by osteoclasts via osteoblasts.

Moreover this investigation shows that glucocorticoids suppressed the production of NO by IL-1. Therefore, glucocorticoids have the capabilities to cause the inhibitory effect of this feed back system towards bone resorption. In this way, glucocorticoids may have been implicated in osteoporosis.

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