FEBS Letters 428 (1998) 212–216 FEBS 20296

# Coexpression of GTP cyclohydrolase I and inducible nitric oxide synthase mRNAs in mouse osteoblastic cells activated by proinflammatory cytokines

Akifumi Togari[a,*], Michitsugu Arai[a], Makio Mogi[a], Ayami Kondo[a], Toshiharu Nagatsu[b]

[a]*Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan*
[b]*Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan*

Received 27 April 1998

**Abstract Proinflammatory cytokines, a combination of IL-1β, TNF-α, and IFN-γ, caused mRNA expression of GTP cyclohydrolase I (GTP-CH), the rate-limiting enzyme in tetrahydrobiopterin ($BH_4$) biosynthesis, and of inducible nitric oxide synthase (iNOS) in a well-characterized osteoblastic clone MC3T3-E1 cell line. We found the expression of the GTP-CH gene in osteoblasts for the first time. The expression of GTP-CH and iNOS mRNAs was found to be maximal at 3 and 9 h, respectively. The expression of both genes elicited increases in $BH_4$ and NO levels. Pharmacological studies using 2,4-diamino-6-hydroxypyrimidine, an inhibitor of GTP-CH activity, showed that $BH_4$ is involved in the activity of iNOS, but not in the induction of iNOS mRNA. The results using an inhibitor of nuclear factor (NF)-κB and activating protein-1 (AP-1) activation suggested that coinduction of the two genes in response to cytokines occurred via activation of NF-κB and AP-1. In MC3T3-E1 cells $BH_4$ and sepiapterin, producing $BH_4$, could protect against apoptosis, i.e. the degradation of nuclear DNA in the cells, induced by NO derived from *S*-nitroso-*N*-acetyl-D,L-penicillamine. These results suggest that the induction of $BH_4$ together with NO by proinflammatory cytokines could protect against NO-induced apoptosis in MC3T3-E1 cells.**



*Key words:* GTP cyclohydrolase I; Inducible nitric oxide synthase; Tetrahydrobiopterin; Nitric oxide; Apoptosis; Osteoblastic cell

## 1. Introduction

GTP cyclohydrolase I (GTP-CH, EC 3.5.4.16) is the first and rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin ($BH_4$). The biosynthesis of $BH_4$ is completed by the subsequent action of 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase. The final product, $BH_4$, serves as a cofactor for the three isoforms of nitric oxide synthase (NOS) [1] and is required for the dimerization and stability of the inducible nitric oxide synthase (iNOS) protein [2–4], and for the protection of NOS activity from inhibition by its product, NO [5].

Since NO has been reported to be released from osteoblast-like cells [6] and to inhibit osteoclastic activity in vitro [6,7], the role of NO as a regulatory molecule in bone has been scrutinized. Recent studies on osteoblast-like cells demonstrated the expression of mRNA, enzyme protein, and enzyme activity of iNOS, which are all induced by proinflammatory cytokines and/or endotoxin among various species, and suggested that osteoblast-derived NO may have an important role in the regulation of cell proliferation and function [8–11]. It is well known that in many other cells, including fibroblasts, brain endothelial cells, vascular smooth muscle cells, and the macrophage cell line RAW264.7, the induction of NO synthesis by proinflammatory cytokines and/or endotoxin is parallel to an enhanced formation of $BH_4$ and an up-regulation of GTP-CH [12–20], and conversely, is suppressed by an inhibitor of GTP-CH [13,15–19]. Thus, $BH_4$ may serve as a cofactor to support the induction of iNOS and the production of NO. Recent observations on vascular smooth muscle that activation of nuclear factor (NF)-κB, which may mediate the induction of NOS [21,22], underlies the induction of GTP-CH gene expression by an immunostimulant [23], suggest that NF-κB may also operate in osteoblasts in controlling GTP-CH gene activity and the expression of the $BH_4$ phenotype.

The aim of the present study was to elucidate the regulation of the GTP-CH and iNOS genes in osteoblasts. We compared the expression of the GTP-CH gene with that of the iNOS gene in response to cytokines, examined the transcriptional mechanism for these inductions, and investigated the physiological significance of coinduction of GTP-CH and iNOS mRNAs. We analyzed the steady-state as well as inducible expression of GTP-CH mRNA in mouse osteoblast-like cells (MC3T3-E1), and examined the effects of NO and $BH_4$ on nuclear DNA fragmentation during these inductions.

## 2. Materials and methods

### 2.1. Materials

MC3T3-E1 cells, established from newborn mouse calvaria by Kodama et al. [24], were kindly supplied by Dr. Amagai (Oou University, Koriyama, Japan). Alpha-modified minimum essential medium (α-MEM) was obtained from Gibco-BRL Laboratories (Grand Island, NY, USA); fetal calf serum (FCS) was from Cell Culture Laboratories (Cleveland, OH, USA). Tissue culture dishes were obtained from Falcon Plastics (Los Angeles, CA, USA). Human recombinant IL-1β, TNF-α, and IFN-γ were from Genzyme (Cambridge, MA, USA). The hemisulfate salt of 2,4-diamino-6-hydroxypyrimidine (DAHP), pyrrolidine dithiocarbamate (PDTC), curcumin and $BH_4$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and L-sepiapterin were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of reagent grade.

### 2.2. Cell culture of MC3T3-E1 cells

Cloned MC3T3-E1 cells were cultured in plastic dishes in α-MEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in air with 5% $CO_2$ and were then subcultured until they were almost confluent. For the experiments to examine gene expression, cells were plated at $10^6$ cells per 10 cm diameter dish and confluent cell cultures were used in all experiments.

*Corresponding author. Fax: (81) (52) 752-5988.
E-mail: togariaf@dpc.aichi-gakuin.ac.jp


*PII* S0014-5793(98)00531-6

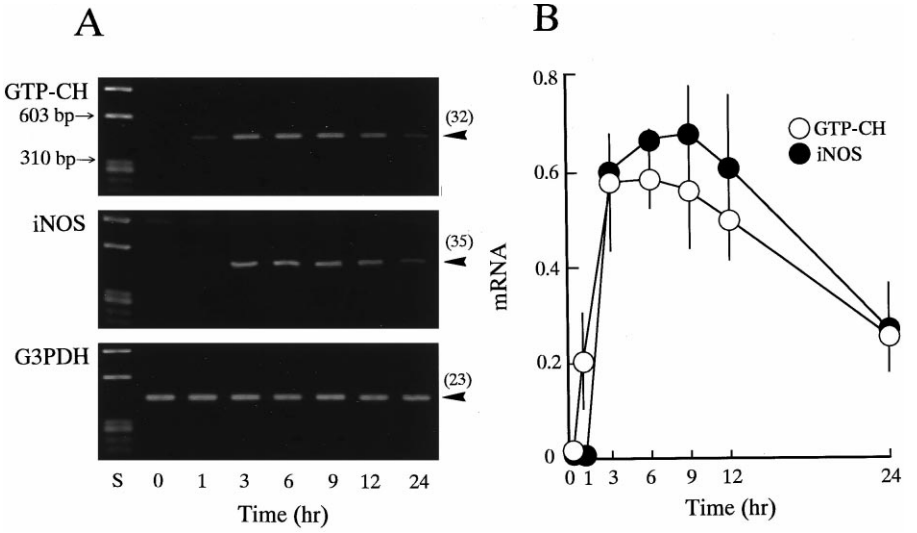


Fig. 1. Time course of the effect of proinflammatory cytokines on coinduction of GTP-CH and iNOS mRNAs in MC3T3-E1 cells. A: RT-PCR analysis for mRNA obtained from cells treated with cytokines (IL-1β 100 U/ml, TNF-α 1 ng/ml, and IFN-γ 200 U/ml) for 0, 1, 3, 6, 9, 12, and 24 h. DNA size markers (φX174/*Hae*III digest) are shown in the left lanes (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrowheads indicate the predicted size of PCR products. B: Relative expression of these inductions. The mRNA level of GTP-CH and iNOS was calculated by dividing the intensity of the GTP-CH and iNOS bands by the intensity of the G3PDH band as determined by a fluoresent image analyzer. Pooled data from four independent experiments are expressed as means ± S.E.M.

*2.3. Analysis of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)*

RNA was extracted from osteoblast-like cells in a 10-cm dish by the guanidinium isothiocyanate method [25]. RT-PCR was performed using standard methods, as previous reported [26]. Briefly, at first cDNA was synthesized using random primers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), followed by PCR amplification using synthetic gene primers specific for mouse iNOS, mouse GTP-CH, and mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) produced from the reported cDNA sequences, respectively [27–29]. The oligonucleotide primers were synthesized on a DNA synthesizer (Expedite model 8909; PerSeptive biosystem, Cambridge, MA, USA), and purified on a polypropylene filter (Oligo Prep kit; Pharmacia Biotech, Uppsala, Sweden). Primers used were as follows: iNOS forward 25-mer, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′; iNOS reverse 25-mer, 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′; GTP-CH forward 21-mer, 5′-TACTCGTCCATTCTGCTCTCG-3′; GTP-CH reverse 21-mer, 5′-GTCTTGCTGTTCATTTTCTGC-3′; G3PDH forward 20-mer, 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH reverse 20-mer, 5′-TCCACCACCCTGTTGCTGTA-3′. PCR amplification was performed with a GeneAmp PCR System (Perkin Elmer/Cetus, Norwalk, CT, USA) according to the following schedule: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min for appropriate cycles. PCR products were electrophoresed on a 2% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, ME, USA) and stained with ethidium bromide. All PCR reactions resulted in the amplification of a single product of the predicted size for iNOS (497 bp), GTP-CH (452 bp), and G3PDH (452 bp), which products were detected with a FluorImager 575 (Molecular Dynamics, Sunnyvale, CA, USA).

*2.4. Determination of nitrite and biopterin*

Quantitative analysis of total biopterin (tetrahydrobiopterin plus oxidized species) was done essentially as described by Fukushima and Nixon [30]. Total NO (nitrite plus nitrate) was determined with a commercially available kit based on the Griess reaction (Cayman Chemical, Ann Arbor, MI, USA).

*2.5. Analysis of DNA fragmentation and cytotoxicity*

Cellular DNA fragmentation assay was performed by the detection of BrdU-labeled DNA fragments in the cytoplasm of cell lysates by solid phase-immobilized anti-DNA MoAb and anti-BrdU MoAb labeled with peroxidase (cellular DNA fragmentation ELISA, Boehringer Mannheim, Mannheim, Germany). In short, MC3T3-E1 cells grown in a culture dish ($2\times10^5$ cells/ml) were labeled with BrdU for 15 h (batch labeling). After labeling, the cell concentration was adjusted to $1\times10^5$ cells/ml, and 100 μl/well were transferred to a microtiter plate (96 wells, flat bottom), and then incubated in the presence of different concentrations of SNAP (0.125–1 mM) mixed with sepiapterin (0.5 mM) for 8 h at 37°C in a humidified atmosphere (5% $CO_2$). The amount of BrdU-labeled DNA released into the cytoplasm of apoptotic cells was quantified by ELISA [31,32].

The cytotoxicity was determined by lactate dehydrogenase (LDH) release measured by a commercially available assay kit (Boehringer Mannheim, Mannheim, Germany). The values (mean ± S.E.M.) for LDH release are presented as the percentage of total LDH (extracellular plus intracellular LDH). Cellular LDH were determined by permeabilizing cells with 1% Triton X-100.

## 3. Results and discussion

This is the first report to demonstrate that osteoblasts are able to express GTP-CH, a rate-limiting enzyme for $BH_4$ synthesis following stimulation by cytokines. We confirmed that iNOS mRNA was also expressed in response to cytokines, as observed in various osteoblast-like cells [8–11]. As shown in Fig. 1, RT-PCR analysis showed that GTP-CH and iNOS mRNAs were not detected at the steady-state but that their level was rapidly increased by exposure of the cells to a combination of IL-1β (100 U/ml), TNF-α (1 ng/ml), and IFN-γ

Table 1
Effect of DAHP on biopterin and NO productions in MC3T3-E1 cells treated with cytokines

| | Biopterin (nmol/well) | NO (pmol/well) |
|---|---|---|
| Control | n.d. | 2.80 ± 0.35 |
| Cytokines | 0.50 ± 0.06 | 4.70 ± 0.24 |
| +DAHP (1.25 mM) | 0.10 ± 0.01 | 3.60 ± 0.35 |
| +DAHP (2.5 mM) | n.d. | 2.50 ± 0.35 |

Biopterin content in cell lysate and NO accumulation in culture medium were detemined at 24 and 48 h, respectively, after exposure to cytokines (IL-1β 100 U/ml, TNF-α 1 ng/ml, and IFN-γ 200 U/ml). DAHP was added 3 h before treatment with cytokines. Each value is the mean ± S.E.M. ($n = 6$). n.d.: not detectable under the present experimental condition.

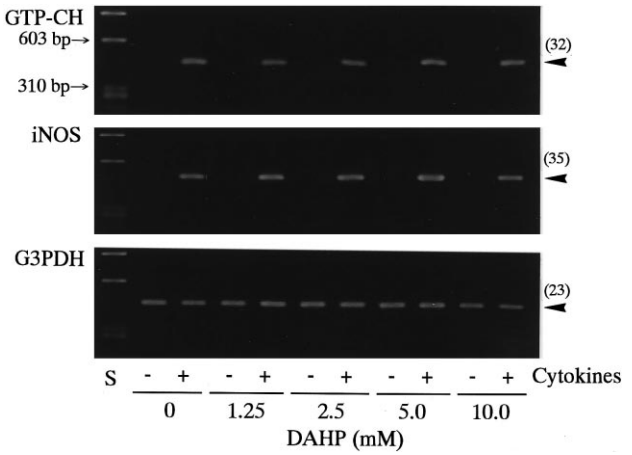


Fig. 2. Effect of DAHP on iNOS and GTP-CH mRNA inductions in MC3T3-E1 cells treated with cytokines. RT-PCR analysis for mRNA obtained from cells treated with DAHP in the presence or absence of cytokines. DAHP (1.25–10 mM) was added 3 h before treatment with cytokines. DNA size markers (φX174/*Hae*III digest) are shown in the left lanes (S). Numbers in parentheses indicate cycles of PCR amplification. Arrowheads indicate the predicted size of PCR products.

(200 U/ml). As shown in Fig. 1B, the expression of GTP-CH and iNOS mRNAs was found to be maximal at 3 and 9 h, respectively, and followed by a decline after 24 h of exposure to the cytokines. Thus, the expression of GTP-CH occurred slightly but substantially prior to the expression of iNOS in response to the cytokines. Individual cytokines had a distinct effect on GTP-CH and iNOS gene expressions. IL-1β (100 U/ml) induced the expression of both genes; TNF-α (1 ng/ml) induced the expression of the GTP-CH gene but not that of the iNOS gene; and IFN-γ (200 U/ml) did not induce the expression of either gene. The gene expression by individual cytokines, IL-1β and TNF-α, was lower than that by cytokine mixtures, IL-1β plus TNF-α plus IFN-γ (data not shown). This may indicate that IFN-γ synergistically augments the effects of IL-1β and/or TNF-α and increases NO and $BH_4$ production in osteoblastic cells.

High-output generation of NO by iNOS and synthesis of $BH_4$ have been demonstrated to be coinduced in mouse fibroblasts, murine brain endothelial cells, and rat vascular smooth muscle cells [12–20]. Conversely, DAHP, which is an inhibitor of GTP-CH activity, reduces both the intracellular $BH_4$ content and the accumulation of NO in the respective cultures [13,15,16]. Furthermore, it has been reported that $BH_4$ is necessary not only for the activity but also for the expression of iNOS in human mesangial cells and that the inhibition of de novo $BH_4$ synthesis with DAHP significantly attenuated iNOS activity as well as mRNA and protein expression in response to IL-1β/TNF-α [33]. These results may indicate that $BH_4$ availability plays an important role in the regulation of iNOS expression. In the present study, we also observed significant increases in total biopterin and NO by cytokines as well as the significant inhibition by DAHP of these increases in osteoblasts, MC3T3-E1 cells (Table 1). As the inhibitory effect of DAHP was at least partially reversed by a precursor of $BH_4$, sepiapterin, we concluded that DAHP downregulates the production of NO via reduced formation of $BH_4$. To examine whether or not biosynthesis of $BH_4$ was necessary to induce iNOS mRNA after exposure to cytokines, we studied the influence of DAHP on expression of iNOS mRNA. As shown in Fig. 2, the induction of iNOS mRNA by cytokines was not affected in mouse osteoblastic cells treated with DAHP (1.25–10 mM).

Recent analyses of promoter sequences showed the presence of cytokine-responsive elements for the binding of transcriptional factors such as activating protein-1 (AP-1) and NF-κB in murine iNOS gene [34], and NF-κB activation is known to be required for cytokine-induced iNOS mRNA expression [21,22]. PDTC inhibits NF-κB activation by suppressing the release of the inhibitory subunit IκB from the latent form of NF-κB [35]; and curcumin inhibits AP-1 binding to its consensus sequence [36]. We tested the effect of PDTC and curcumin on the expression of iNOS and GTP-CH mRNAs in

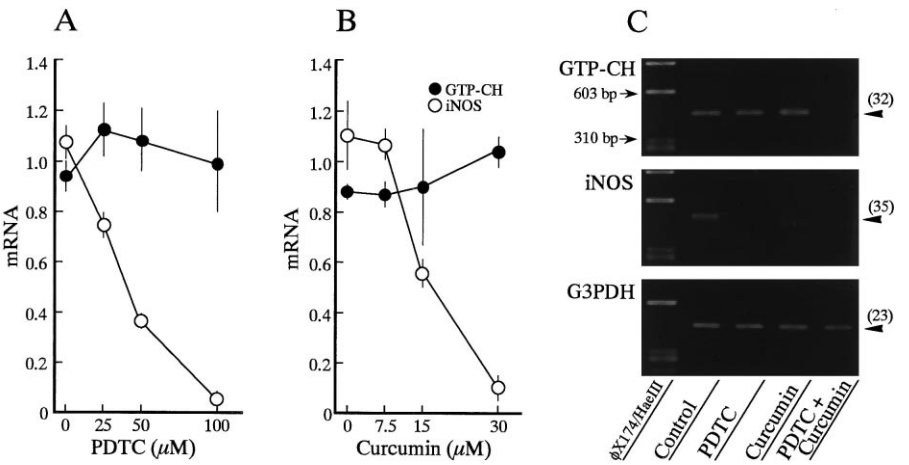


Fig. 3. Effects of PDTC and curcumin on induction of GTP-CH and iNOS mRNAs in MC3T3-E1 cells treated with cytokines. A: Effects of PDTC (25–100 μM) on induction of GTP-CH (●) and iNOS (○) mRNAs by cytokines. Relative expression of these inductions. The mRNA level of GTP-CH and iNOS was calculated by dividing the intensity of the GTP-CH and iNOS bands by the intensity of the G3PDH band as determined by a fluoresent image analyzer. Pooled data from three independent experiments are expressed as means ± S.E.M. B: Effects of curcumin (7.5–30 μM) on induction of GTP-CH (●) and iNOS (○) mRNAs by cytokines. Pooled data from three independent experiments are expressed as means ± S.E.M. C: Agarose gel electrophoresis of GTP-CH and iNOS mRNAs in cells treated with PDTC and/or curcumin. PDTC (50 μM) and/or curcumin (30 μM) were added 3 h before treatment with cytokines. DNA size markers (φX174/*Hae*III digest) are shown in the left lanes (S). Numbers in parentheses indicate cycles of PCR amplification. Arrowheads indicate the predicted size of PCR products.

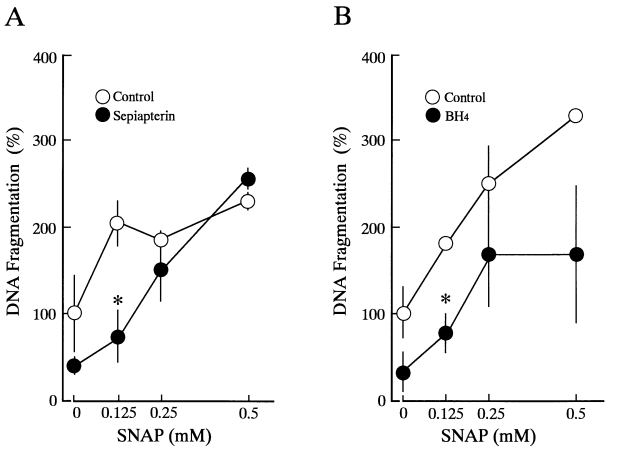


Fig. 4. Effect of sepiapterin (A) and $BH_4$ (B) on DNA fragmentation elicited by SNAP. Cells were incubated in the presence of SNAP (0.125–0.5 mM) with or without sepiapterin (0.5 mM) or $BH_4$ (1 mM) for 8 h. The amount of BrdU-labeled DNA released into the cytoplasm of apoptotic cells was quantified and expressed as a percentage of the control. Each value is the mean ± S.E.M. ($n = 4$). (*$P < 0.05$, statistical difference from control, by Student's $t$-test).

osteoblastic cells (Fig. 3). The cytokine-induced expression of iNOS mRNA was abolished by either PDTC (50 μM) or curcumin (30 μM), suggesting that both NF-κB and AP-1 activation may be required for the cytokine-induced expression of the iNOS gene. On the other hand, the expression of GTP-CH mRNA induced by cytokines was not abolished by either PDTC or curcumin, but was abolished by PDTC plus curcumin. This finding suggests that dual operation of NF-κB stimulation and AP-1 activation is essential for the induction of cytokine-induced expression of the GTP-CH gene. Thus, these pharmacological experiments indicated that the activation of NF-κB and AP-1 may be involved in the expression of both iNOS and GTP-CH mRNAs in osteoblastic cells. The inhibitory effect of PDTC and/or curcumin on gene expression induced by combined cytokines was also observed on gene expression by individual cytokines (data not shown).

It is an interesting problem to elucidate the physiological meaning of the coinduction of iNOS and GTP-CH genes in response to cytokines in mouse osteoblastic cells. There are some data suggesting physiological significance for the coinduction, which increases the NO as well as $BH_4$ content in the cells. In osteoblastic cells, cytokine-induced increases in NO production have been reported to be associated with a marked inhibition of proliferation [8,9]. This inhibition was reversed by abrogation of NO synthesis with the competitive inhibitor of NO synthase under these conditions, suggesting that NO may be responsible for the inhibition of osteoblastic proliferation induced by these cytokines (the combination of IL-1β, TNF-α and IFN-γ). Recently, it was demonstrated that elevating intracellular $BH_4$ levels significantly enhanced the proliferation of rat PC12 cells, rat C6 glioma, and human fibroblasts above basal proliferation rates [37]. These findings suggest that the action of NO on cell proliferation is the opposite of that of $BH_4$. To determine the physiological interaction between $BH_4$ and NO in osteoblastic cells, we examined the effects of these products on nuclear DNA degradation, which is regarded as a hallmark of apoptotic cell death [38], in light of the recent observation of NO-induced apoptosis in osteoblastic cells [39]. As shown in Fig. 4, sepiapterin (0.5 mM), known to produce $BH_4$ through the pterin salvage pathway within cells, as well as $BH_4$ (1 mM) significantly inhibited DNA fragmentation induced by SNAP (0.125 mM), an NO generator. In the cells showing DNA fragmentation by treatment of SNAP (0.125 mM) for 8 h, no significant increase in LDH release was observed (basal LDH release 18.0 ± 2.5%, SNAP-induced LDH release 16.5 ± 2.5%), suggesting that necrotic cell death, if present, represents a minor pathway of SNAP-induced cytotoxicity in MC3T3-E1 cells. This result indicates that $BH_4$ may protect against the degradation of nuclear DNA induced by NO, suggesting physiological antagonism for cell viability by these products.

In conclusion, the present findings indicate that coinduction of iNOS and GTP-CH mRNAs in response to cytokines, which elicited increases in NO and $BH_4$ levels, occurs probably via NF-κB and AP-1 activation in mouse osteoblastic cells and that $BH_4$ is involved in the activity of iNOS, but not in the induction of iNOS mRNA. As a physiological significance of gene expression of GTP-CH with iNOS induced by proinflammatory cytokines, we propose that the induction of $BH_4$ synthesis due to GTP-CH gene expression may have protective activity against NO-induced apoptosis.

*Acknowledgements:* The excellent technical assistance of Yoko Ishikawa and Shoko Imamura is gratefully acknowledged. This study was supported in part by a Grant-in-Aid to A. Togari (09671912) from the Ministry of Education, Science, Sports, and Culture of Japan, and by the Suzuken Memorial Foundation.

## References

[1] Moncada, S., Palmer, R.M. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–142.
[2] Giovanelli, J., Campos, K.L. and Kaufman, S. (1991) Proc. Natl. Acad. Sci. USA 88, 7091–7095.
[3] Hevel, J.M. and Marletta, M.A. (1992) Biochemistry 31, 7160–7165.
[4] Baek, K.J., Thiel, B.A., Lucas, S. and Stuehr, D.J. (1993) J. Biol. Chem. 268, 21120–21129.
[5] Griscavage, J.M., Fukuto, J.M., Komori, Y. and Ignarro, L.J. (1994) J. Biol. Chem. 269, 21644–21649.
[6] Lowik, W.G.M.C., Nibbering, P.H., van de Ruit, M. and Papapoulos, S.E. (1994) J. Clin. Invest. 93, 1465–1472.
[7] MacIntyre, I., Zaidi, M., Alam, A.S.M.T., Datta, H.K., Moonga, B.S., Lidbury, P.S., Hecker, M. and Vane, J.R. (1991) Proc. Natl. Acad. Sci. USA 88, 2936–2940.
[8] Ralston, S.H., Todd, D., Helfrich, M., Benjamin, N. and Grabowski, P.S. (1994) Endocrinology 135, 330–336.
[9] Hukkanen, M., Hughes, F.J., Buttery, L.D.K., Gross, S.S., Evans, T.J., Seddon, S., Riveros-Moreno, U., MacIntyre, I. and Polak, J.M. (1995) Endocrinology 136, 5445–5453.
[10] Riancho, J.A., Salas, E., Zarrabeitia, M.T., Olmos, J.M., Amado, J.A., Fernandez-Luna, J.L. and Gonzalez-Macias, J. (1995) J. Bone Mineral Res. 10, 439–446.
[11] Riancho, J.A., Zarrabeitia, M.T., Fernandez-Luna, J.L. and Gonzalez-Macias, J. (1995) Mol. Cell. Endocrinol. 107, 87–92.
[12] Werner, E.R., Werner-Felmayer, G., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1989) Biochem. J. 262, 861–866.
[13] Werner-Felmayer, G., Werner, E.R., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1990) J. Exp. Med. 172, 1599–1607.
[14] Fleming, I., Gray, G.A., Schott, C. and Stoclet, J.C. (1991) Eur. J. Pharmacol. 200, 375–376.
[15] Gross, S.S., Jaffe, E.A., Levi, R. and Kilbourn, R.G. (1991) Biochem. Biophys. Res. Commun. 178, 823–829.
[16] Gross, S.S. and Levi, R. (1992) J. Biol. Chem. 267, 25722–25729.

[17] Jorens, P.G., van Overveld, F.J., Bult, H., Vermeire, P.A. and Herman, A.G. (1992) Br. J. Pharmacol. 107, 1088–1091.
[18] Sakai, N., Kaufman, S. and Milstien, S. (1993) Mol. Pharmacol. 43, 6–10.
[19] Schoedon, G., Schneemann, M., Hofer, S., Guerrero, L., Blau, N. and Schaffner, A. (1993) Eur. J. Biochem. 213, 833–839.
[20] Werner, E.R., Werner-Felmayer, G. and Wachter, H. (1993) Proc. Soc. Exp. Biol. Med. 203, 1–12.
[21] Xie, Q.W., Kashiwabara, Y. and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708.
[22] Saldeen, J. and Welsh, N. (1994) Biochem. Biophys. Res. Commun. 203, 149–155.
[23] Hattori, Y., Nakanishi, N., Kasai, K., Shimoda, S. and Gross, S.S. (1996) Eur. J. Pharmacol. 296, 107–112.
[24] Kodama, H., Amagai, Y., Sudo, H., Kasai, S. and Yamamoto, S. (1981) Jpn. J. Oral Biol. 23, 899–901.
[25] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[26] Togari, A., Ichinose, H., Matsumoto, S., Fujita, K. and Nagatsu, T. (1992) Biochem. Biophys. Res. Commun. 187, 359–365.
[27] Nomura, T., Ichinose, H., Sumi-Ichinose, C., Nomura, H., Hagino, Y., Fujita, K. and Nagatsu, T. (1993) Biochem. Biophys. Res. Commun. 191, 523–527.
[28] Lyons, C.R., Orloff, G.J. and Cunningham, J.M. (1992) J. Biol. Chem. 267, 6370–6374.
[29] Arcari, P., Martinelli, R. and Salvatore, F. (1984) Nucleic Acids Res. 12, 9179–9189.
[30] Fukushima, T. and Nixon, J.C. (1980) Anal. Biochem. 102, 176–188.
[31] Magaud, J.P., Sargent, I. and Mason, D.Y. (1988) J. Immunol. Methods 106, 95–100.
[32] Muir, D., Varon, S. and Manthorpe, M. (1990) Anal. Biochem. 185, 377–382.
[33] Saura, M., Perez-Sala, D., Canada, F.J. and Lamas, S. (1996) J. Biol. Chem. 271, 14290–14295.
[34] Xie, Q.W., Whisnant, R. and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784.
[35] Schreck, R., Meier, B., Mannel, D.N., Droge, W. and Baeuerle, P.A. (1992) J. Exp. Med. 175, 1181–1194.
[36] Hung, T.S., Lee, S.C. and Lin, J.K. (1991) Proc. Natl. Acad. Sci. USA 88, 5292–5296.
[37] Anastasiadis, P.Z., States, J.C., Imerman, B.A., Louie, M.C., Kuhn, D.M. and Levine, R.A. (1996) Mol. Pharmacol. 49, 149–155.
[38] Henkart, P.A. (1995) J. Immunol. 154, 4905–4908.
[39] Damoulis, P.D. and Hauschka, P.V. (1997) J. Bone Mineral Res. 12, 412–422.