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Involvement of Nitric Oxide and Biopterin in Proinflammatory Cytokine-Induced Apoptotic Cell Death in Mouse Osteoblastic Cell Line MC3T3-E1

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ABSTRACT. We previously demonstrated that the addition of proinflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and interferon-γ) caused induction of mRNAs for inducible nitric oxide (NO) synthase and GTP cyclohydrolase I, a rate-limiting enzyme for 5,6,7,8-tetrahydrobiopterin (BH_4) biosynthesis, and produced their end-products, NO and BH_4, in osteoblastic cells. In the present study, we examined whether NO and BH_4, biologically active substances produced in response to proinflammatory cytokines, are involved in the effect of these cytokines on cell viability and apoptotic cell death involving DNA fragmentation. Cytokines as well as *S*-nitroso-*N*-acetyl-*d*,*l*-penicillamine, an NO generator, decreased cell viability, whereas sepiapterin, which was converted intracellularly to BH_4, increased it. The examination of cytotoxicity measured in terms of lactate dehydrogenase release and apoptotic cell death assessed by flow cytometric analysis showed that cytokine-induced reduction of cell viability may be based upon cell death by apoptosis, but not lytic death as in necrosis. In the presence of sepiapterin, cytokine treatment resulted in a statistically pronounced reduction in the amount of DNA fragmentation. Furthermore, this fragmentation could be blocked by 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, an NO scavenger. These results suggest that cytokine-induced apoptotic cell death is attributed to NO and is protected by BH_4, and that osteoblastic cells in response to proinflammatory cytokines operate both a stimulatory process resulting in NO production and an inhibitory one resulting in BH_4 production for apoptotic cell death. Cytokine-induced apoptotic cell death may be a consequence of the predominance of the stimulatory process over the inhibitory process. BIOCHEM PHARMACOL **58**;4:649–654, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. tetrahydrobiopterin; nitric oxide; cytokine; NO scavenger; sepiapterin; apoptosis

In the bone microenvironment, the dynamic balance between resorption and formation maintains skeletal homeostasis. The cells responsible for these functions, osteoclasts and osteoblasts, are regulated by many different mediators, including osteotrophic hormones and proinflammatory cytokines. Of the latter, TNF-α† and IL-1β are mediators of inflammatory bone loss [1], whereas IFN-γ has been reported to selectively inhibit cytokine-induced bone resorption [2]. In most cases, the primary target cell for these cytokines is the osteoblast, which in turn regulates osteoclast precursor recruitment and/or activation [3]. A multitude of effects have been attributed to the action of one or more of these cytokines in regulating the proliferation and phenotypic characteristics of osteoblasts from various species [4–6]. Very recently, proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ were shown to induce apoptosis in mouse osteoblasts [7, 8].

Previously, we demonstrated that a mixture of TNF-α, IL-1β, and IFN-γ caused mRNA expression of GTP-CH, the first and rate-limiting enzyme in BH_4 biosynthesis, as well as that of inducible NO synthase in the well-characterized clonal osteoblastic MC3T3-E1 cell line [9]. Their gene expression, followed by an increase in NO and BH_4 levels, was proved to occur via nuclear factor-κB and activating protein-1 activation by use of inhibitors of nuclear factor-κB and activating protein-1 activation. Furthermore, we found that exogenous BH_4 protected against apoptosis, i.e. the degradation of nuclear DNA in the cells, induced by NO derived from SNAP, an NO generator. From these results, we suggested that the induction of BH_4 together with NO by proinflammatory cytokines could protect against NO-induced apoptosis in MC3T3-E1 cells.

To confirm whether both BH_4 and NO act in conjunction with proinflammatory cytokines affecting cell proliferation and apoptotic cell death in the mouse osteoblastic

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† *Abbreviations:* NO, nitric oxide; GTP-CH, GTP cyclohydrolase I; BH_4, 5,6,7,8-tetrahydrobiopterin; SNAP, *S*-nitroso-*N*-acetyl-*d*, *l*-penicillamine; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; LDH, lactate dehydrogenase; MoAb, monoclonal antibody; BrdU, 5-bromo-2′-deoxyuridine; and carboxy-PTIO, 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide.

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cell line MC3T3-E1, we examined pharmacologically the involvement of NO and BH_4 in cytokine-induced cell death. This present study showing that the apoptotic cell death elicited by proinflammatory cytokines is based on endogenously elevated NO and is protected by intracellularly elevated BH_4 supports our previous demonstration [9].

MATERIALS AND METHODS

Cell Culture

MC3T3-E1 cells established from new-born mouse calvaria are a clonal osteogenic cell line whose cells differentiate into osteoblasts, and are frequently used as a model to study the effects of proinflammatory cytokines *in vitro* [8, 10]. We cultivated these cells in plastic dishes containing α-minimal essential medium, and collected conditioned medium at various time points or following treatment with various doses of cytokines for total NO assay, LDH assay, biopterin determination, and cellular DNA fragmentation assay by ELISA. Human recombinant TNF-α, IL-1β, and IFN-γ were from Genzyme. The cytokine mixture (1 U) consisted of 1 ng/mL of TNF-α, 100 IU/mL of IL-1β, and 200 IU/mL of IFN-γ. We utilized, in general, 5 U of cytokines to induce apoptotic cell death, and the concentrations of cytokine mixture seem rather high compared with the range of effectiveness published for various similar studies using MC3T3-E1 [8, 10, 11]. Damoulis and Hauschka [8] demonstrated that human cytokines, especially in TNF-α, had a significantly lower cytotoxic effect than murine cytokines for MC3T3-E1 cells. Therefore, high contents of cytokines are needed to induce DNA fragmentation in current mouse osteoblastic cells. BH_4 was obtained from Sigma. Sepiapterin was purchased from Schircks Laboratory and 2,4-diamino-6-hydroxypyrimidine from Aldrich Chem. Co. SNAP and carboxy-PTIO were purchased from Dojindo Co. Carboxy-PTIO is a relatively stable compound and exhibits low toxicity compared with l-arginine analogs [12].

Determination of Cell Proliferation (Cell Viability) and Cytotoxicity

Cell number was assessed by the use of a colorimetric proliferation assay (cell counting kit: Dojindo Co). MC3T3-E1 cells were cultured in 96-well plates (10,000 cells/well). After incubation of cells in each well under the appropriate test condition, the medium was removed and replaced with medium containing WST-1 dye. After a 2-hr incubation at 37°, absorbance was measured at 450 nm with a reference wavelength of 620 nm, and the data were expressed as cell viability (% of untreated control). The cytotoxicity of cytokines was determined by LDH release measured by a commercially available LDH assay kit (Boehringer Mannheim). The percentage of LDH release was calculated by the following formula: % LDH release = LDH released in conditioned medium/(LDH released in conditioned medium + LDH in cells lysed by Triton-X100 [1%]). Differences between control and experimental values were subjected to statistical analysis by Student's *t*-test.

Determination of Apoptotic Cell Death by ELISA

The cellular DNA fragmentation assay was performed by the detection of BrdU-labeled DNA fragments in the cytoplasm of cell lysates by use of solid phase-immobilized anti-DNA MoAb and anti-BrdU MoAb labeled with peroxidase (cellular DNA fragmentation ELISA, Boehringer Mannheim). In short, MC3T3-E1 cells grown in culture dishes (2×10^5 cells/mL) were labeled with BrdU for 15 hr; the cell concentration was then adjusted to 1×10^5 cell/mL and 100 μL of suspension was transferred to each well of a microtiter plate (96 wells, flat bottom). The cells were then incubated in the presence of different concentrations of the cytokine mixture along with sepiapterin (125–1000 μM) for 8 hr at 37° in a humidified atmosphere (5% CO_2). The amount of BrdU-labeled DNA released into the cytoplasm of apoptotic cells was quantified by ELISA [13].

Evaluation of Cellular Apoptosis by Flow Cytometric Analysis

MC3T3-E1 cells (1×10^6 cells) exposed to modifiers were harvested, fixed, treated with RNase-A solution, and stained with a nuclear staining solution (propidium iodine). The DNA content in each cell-cycle phase was determined by FACS Calibur (Becton Dickinson). The number of apoptotic cells was presented as a percentage of hypodiploid nuclei.

Determination of Total NO and Biopterin

MC3T3-E1 cells were plated in 6-well plates (2×10^5 cells/well) and incubated for 24 hr in the presence of various cytokine mixtures before determination of NO and BH_4. Subsequently, the conditioned media were collected for determination of NO, and the cells were then homogenized by 0.1 N perchloric acid containing 1 mM dithiothreitol. Quantitative analysis of total biopterin (BH_4 plus oxidized species) was done essentially as described by Fukushima and Nixon [14]. Total NO (nitrite plus nitrate) was determined with a commercially available kit based on the Griess reaction (Cayman Chemical).

RESULTS

The treatment with the cytokine mixture, which elicited marked elevation of endogenous BH_4 and NO contents in MC3T3-E1 cells [9], caused a potent concentration-dependent inhibition of cell proliferation (Fig. 1A). The incubation with freshly prepared SNAP (16–1000 μM), a commonly used NO generator, caused a marked decrease in cell viability (Fig. 1C). In contrast, sepiapterin (16–500 μM), which provides BH_4 through the pterin salvage pathway within cells, caused a concentration-dependent increase in

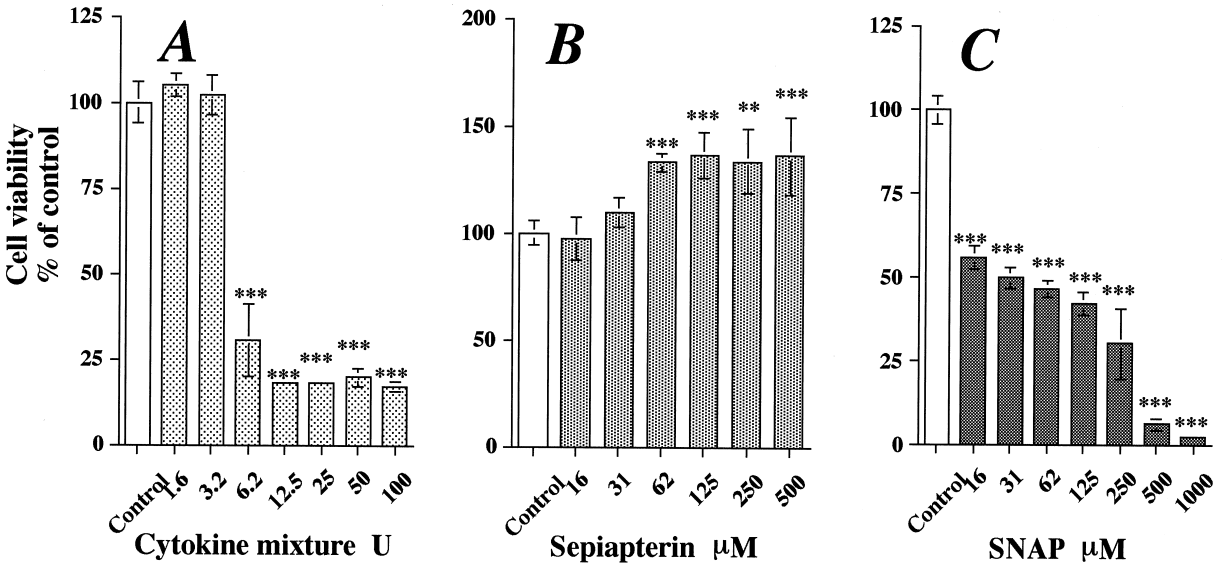

FIG. 1. Effects of cytokine mixture (A), sepiapterin (B), and SNAP (C) on the viability of MC3T3-E1 cells. MC3T3-E1 cells were plated in 96-well plates (1×10^4 cells/well) and incubated for 24 hr in the presence of various modifiers before determination of cell viability by the colorimetric assay (WST-1). The cytokine mixture (1 U) consisted of 1 ng/mL of TNF-α, 100 IU/mL of IL-1β, and 200 IU/mL of IFN-γ. Cell viability is expressed as a % of the control. Values represent the mean ± standard error of six independent determinations. Significantly different from the control, **$P<0.01$, ***$P<0.005$.

MC3T3-E1 cell proliferation to 137% of the control (Fig. 1B); however, 2,4-diamino-6-hydroxypyrimidine (20 mM), an inhibitor of GTP-CH, resulted in a decrease in cell viability to 52% of the control (data not shown). Furthermore, treatment with the cytokine mixture at 3U, which caused no decrease in cell viability (Fig. 1A), caused a significant increase in endogenous biopterin content (2.58-fold) and NO production (2.65-fold) in MC3T3-E1 cells (Table 1). A large amount of cytokines (10 or 30 U), which caused a slight increase in biopterin production (3.84- or 4.50-fold) and a great increase in NO production (10.24- or 20.10-fold), caused a great decrease in cell viability (Fig. 1A). To determine whether the cell viability decreased by the cytokine mixture could be due to apoptotic cell death, we monitored three parameters of the process, namely, LDH release as a measure of cytotoxicity, DNA fragmentation, and FACS analysis. The cytokine mixture was not cytotoxic as assessed by a commercial assay for cytotoxicity based upon release of LDH, for the cells exposed to the modifiers (cytokine mixture) released an amount of LDH (17.0 ± 3.5%) that was similar to that released by control cells (14.4 ± 2.2%), indicating the relative absence of lytic death as occurs in necrosis. The cellular DNA fragmentation assay by ELISA demonstrated that the cytokines potently elevated DNA fragmentation in a time-dependent manner over the first 7 hr, which was followed by a gradual, continuous increase until 22 hr (Fig. 2). Induction of

TABLE 1. Total NO and biopterin content in MC3T3-E1 cells treated with the cytokine mixture

	Total NO (nmol/dish)	Biopterin (nmol/dish)
Control	1.42 ± 0.15 (100)	0.62 ± 0.06 (100)
Cytokine mixture		
1U	2.44 ± 0.19* (168)	1.15 ± 0.03* (185)
3U	3.84 ± 0.26† (265)	1.60 ± 0.03† (258)
10U	14.85 ± 1.84† (1024)	2.40 ± 0.03† (387)
30U	29.14 ± 1.83† (2010)	2.79 ± 0.08† (450)

Each value represents the mean ± SEM, and % of the control is shown in parentheses. One unit (U) of the cytokine mixture consisted of 1 ng/ml of TNF-α, 100 IU/ml of IL-1β, and 200 IU/ml of IFN-γ. Significantly different from the control: *$P<0.01$; †$P<0.005$.

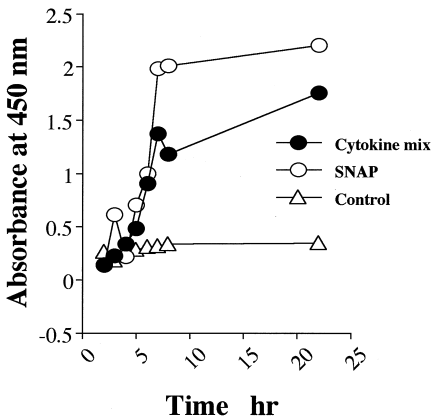

FIG. 2. Time dependency of the cellular DNA fragmentation in MC3T3-E1 cells induced by cytokines or SNAP. The cellular DNA fragmentation assay was performed by the detection of BrdU-labeled DNA fragments in the cytoplasm of cell lysates using solid phase-immobilized anti-DNA MoAb and anti-BrdU MoAb labeled with peroxidase. After the indicated time of incubation in the presence of the cytokine mixture (5 U) or SNAP (100 μM), the amount of BrdU-labeled DNA released into the cytoplasm of the cells was quantified by ELISA. Data shown are representative of three independent experiments.

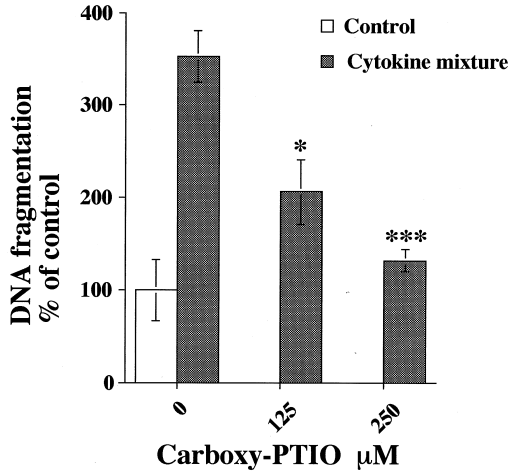

FIG. 3. Carboxy-PTIO blocks cytokine-induced DNA fragmentation in MC3T3-E1 cells. BrdU-labeled MC3T3-E1 cells were plated in 96-well plates and incubated for 24 hr in the presence of various concentrations of carboxy-PTIO (NO scavenger, 125 or 250 μM) combined with the cytokine mixture (5 U) before determination of BrdU-labeled DNA released into the cytoplasm of the apoptotic cells, which was quantified by ELISA. Values represent at the % of control and mean ± standard error of four independent determinations. Significantly different from cytokine mixture without carboxy-PTIO *$P<0.05$, ***$P<0.005$.

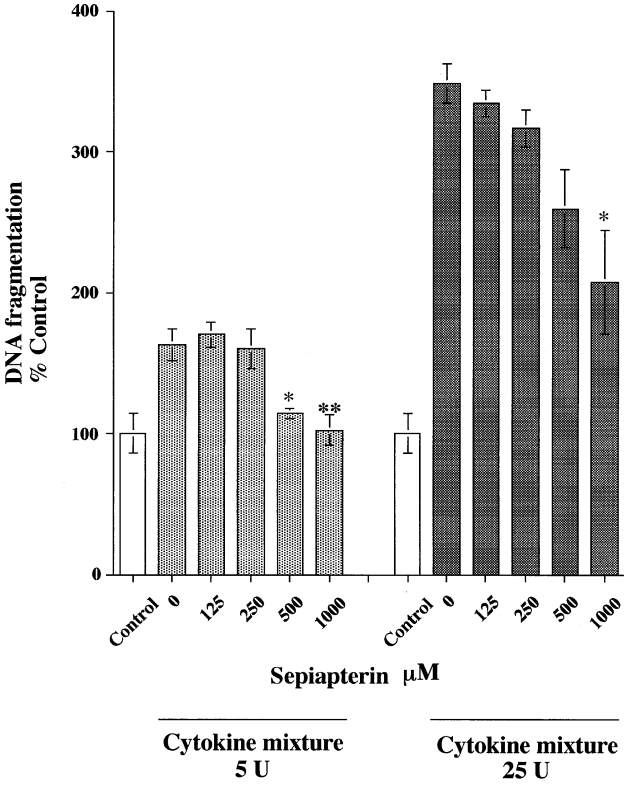

FIG. 4. Cellular DNA fragmentation in cells induced by the cytokine mixture combined with sepiapterin. BrdU-labeled MC3T3-E1 cells were plated in 96-well plates and incubated for 24 hr in the pressure of various concentrations of sepiapterin and cytokine mixture (5 or 25 U). Then, BrdU-labeled DNA released into the cytoplasm of the cells was determined and quantified by ELISA. Values represent the mean ± standard error of four independent determinations. Significantly different from sepiapterin at 0 μM, *$P<0.05$, **$P<0.01$.

apoptosis in MC3T3-E1 cells was also examined by measuring the decrease in cellular DNA content as revealed by flow cytometric analysis. In untreated control cultures, percentage of apoptosis was less than 5% after a 22-hr incubation. In comparison with control cultures, those incubated with the cytokine mixture showed a significant increase in apoptosis up to 41% (data not shown). Therefore, these experiments demonstrated that the cytokine mixture caused cell death in MC3T3-E1 mediated by apoptosis.

To prove the physiological involvement of NO and BH_4 in the cytokine-induced apoptosis, we examined the effect of carboxy-PTIO, a specific scavenger of the NO radical, and that of sepiapterin on apoptotic cell death in MC3T3-E1 cells treated with cytokines. Carboxy-PTIO (125 or 250 μM) could block the DNA fragmentation induced by the cytokine mixture, suggesting that the NO radical triggered the DNA fragmentation (Fig. 3). Moreover, cytokine-induced DNA fragmentation was blocked by sepiapterin in a dose-dependent manner (Fig. 4).

DISCUSSION

It is well known that BH_4 serves as a cofactor for the three isoforms of NO synthase [15] and is required for the dimerization and stability of the inducible nitric oxide synthase protein [16–18], and for the protection of NO synthase activity from inhibition by its product, NO [19]. Previous studies have shown that NO and BH_4 synthesis are co-induced by cytokine treatment and that inhibition of BH_4 biosynthesis results in a complete blockage of NO production in murine fibroblasts [20], macrophages [20, 21], smooth muscle cells [22], endothelial cells [23], and C6 glioma cells [24]. However, up to now there has been no report focusing on the modulating and competitive function of endogenous BH_4 against NO-mediated toxicity. Our current data demonstrate the novel physiological significance of BH_4, i.e. its antiapoptotic function against cytotoxicity. As shown in Fig. 1, the cytokine mixture as well as SNAP caused a marked decrease in cell viability, suggesting that the antiproliferative effect of cytokines may be based upon endogenously elevated NO, which has been demonstrated to be induced by proinflammatory cytokines [9]. On the other hand, a slight but substantial increase in cell viability was observed by intracellularly elevated BH_4, derived from sepiapterin. The mitogenic effect of BH_4 was also demonstrated in rat PC12 cells [25, 26] and HL-60 cells [27]. Because not only NO but also BH_4 has been reported to be induced by proinflammatory cytokines in MC3T3-E1 cells [9], both the antiproliferative effect of NO and the proliferative effect of BH_4 may be operative in MC3T3-E1 cells treated with the cytokines. Furthermore, our present data show that the effect of NO on cell viability is greater than that of BH_4 in cells treated with a high concentration

of cytokines. This finding demonstrates that the elevation of NO and BH_4 is commonly involved in the cell viability of MC3T3-E1 cells exposed to proinflammatory cytokines, and that the dual levels of activation of and protection against cell death determine the degree of cell viability of cytokine-treated cells. Therefore, the current results indicate that the elevation of NO by cytokines may be involved in apoptotic cell death in MC3T3-E1 cells and that the elevation of BH_4 by cytokines may protect against the degradation of nuclear DNA induced by NO, suggesting the production of physiologically antagonistic compounds for cell viability by cytokines in MC3T3-E1 cells. This is a reasonable strategy for host defense against NO toxicity, because the BH_4 biosynthetic pathway appears to be constitutively active in all cells except for GTP-CH [20]. The data in Table 1 and Fig. 4 indicate that cytokines could produce low amounts of BH_4, and the high concentration of BH_4 was needed to prevent apoptosis. However, it is possible that the accumulation of BH_4 results in a high concentration of BH_4 at the microenvironmental site (e.g. at sites of inflammation).

In conclusion, cytokine-induced apoptotic cell death in MC3T3-E1 cells is attributable to endogenously elevated NO and is protected by intracellularly elevated BH_4, which is induced together with NO by proinflammatory cytokines. Thus, BH_4 may be the counterpart and counterbalance to NO toxicity in MC3T3-E1 cells. Osteoblastic cells in response to proinflammatory cytokines operate both stimulatory and inhibitory processes for apoptotic cell death, and a predominance of the stimulatory process results in apoptotic cell death.

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