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VITAMIN K_2 (MENATETRENONE) INHIBITS PROSTAGLANDIN SYNTHESIS IN CULTURED HUMAN OSTEOBLAST-LIKE PERIOSTEAL CELLS BY INHIBITING PROSTAGLANDIN H SYNTHASE ACTIVITY

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Abstract—Prostaglandin (PG) E_2, a potent bone-resorbing agent, is synthesized in osteoblast-like cells. Since vitamin K reportedly plays an important role in bone metabolism, we investigated the effects of vitamin K_2 (menatetrenone) on PGE_2 production by human osteoblast-like periosteal cells. In cells incubated with menatetrenone ($1\ \mu g/mL = 2.25 \times 10^{-6}$ M) for 2 days, PGE_2 production was reduced to 50% of that in untreated control cells. This inhibition was dose and time dependent for up to $10\ \mu g$/mL and 20 days, respectively, and involved two major steps. In one of these menatetrenone at doses of 0.5–$10\ \mu g$/mL dose dependently inhibited the calcium ionophore A23187-induced release of arachidonic acid (AA) from membrane phospholipids, and in the other the conversion of AA to PG was inhibited, as evidenced by the PG-synthesizing activity in the homogenates of menatetrenone-treated cells with AA being lower than that in untreated cells. The inhibitory effect was almost identical to that for PG production. The PG synthesizing activity in cell homogenates was inhibited only by a high concentration of menatetrenone ($10\ \mu g$/mL) when this was added directly. Menatetrenone ($1\ \mu g$/mL) also inhibited 52% of the purified PGH synthase activity from a ram seminal vesicle. This study shows that menatetrenone inhibited PGE_2 release from cells by inhibiting both PG production steps, AA release from the membrane and PG synthesizing activity with AA. Inhibition of PGE_2 production by menatetrenone might be important in improving bone metabolism.

Vitamin K, a cofactor of γ-carboxylase, is distributed throughout most body tissue [1, 2]. In bone, a vitamin K-dependent Gla protein, osteocalcin, is important in mineralization [3–5]. In fact, vitamin K has been shown to be closely involved in bone metabolism. Vitamin K_2 stimulates fracture healing [6]; the circulating level of vitamin K_1 in patients with a fractured neck of the femur was found to be lower than that in age-matched healthy persons [7]. Vitamin K_2 with four unsaturated prenyl side chains (menatetrenone) enhances mineralization in human osteoblasts *in vitro* (unpublished findings); and vitamin K_2 inhibits bone resorption in mouse calvarias [8]. Since prostaglandin (PG‡) E_2 is a powerful bone resorbing agent [9], it is conceivable that PG might be involved in the effects manifested by menatetrenone.

The inhibitory effects of vitamin K_1 (phylloquinone) and K_3 (menadione) on PG production have been reported. The underlying mechanism was revealed in cultured endothelial cells, in which phylloquinone did not inhibit PGH synthase (PHS) activity, but inhibited arachidonic acid (AA) release from membrane phospholipids (namely, phospholipase) [10]. Menadione inactivates PGI_2 synthase via a reactive species resulting from its metabolism by endothelial cells, and does not directly affect either the conversion of AA to PG or the release of AA from membranes [11].

The effects of menatetrenone, a biologically-active vitamin K_2 analog, on PG synthesis have not yet been reported. We found that this agent inhibited PG production in human osteoblast-like periosteal cells. We describe the mechanism responsible, i.e., the inhibition of both the release of AA from the membrane and PG synthesis from AA. Although a hydroperoxide intermediate is involved in vitamin K-dependent carboxylation [12], we found the hydroperoxide was not generated after the cells were exposed to menatetrenone. We discuss the possibility that the more powerful inhibitory effect found in menatetrenone pre-treated cells than in cell homogenates is due to menatetrenone-induced proteins.

MATERIALS AND METHODS

Materials. Menatetrenone (2-methyl-3-tetraprenyl-1,4-naphthoquinone) was chemically synthesized by the Eisai Pharmaceutical Co. (Tokyo, Japan). The purity and quality were confirmed by HPLC and NMR; the purity was 99.8%. Radioimmunoassay (RIA) kits for [^{125}I]PGE_2 and

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‡ Abbreviations: AA, arachidonic acid; α-MEM, α-minimum essential medium; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGE_2 and PGH_2, prostaglandin E_2 and H_2; RIA, radioimmunoassay; α-GP, disodium α-glycerophosphate; FBS, fetal bovine serum; PBS(−), Ca^{2+} and Mg^{2+}-free phosphate-buffered saline; PHS, prostaglandin H synthase.

[^{125}I]6-keto-PGF$_{1\alpha}$ (6 keto-prostaglandin F$_{1\alpha}$) and [^{14}C]AA (sp. act. 52.0 mCi/mmol), were purchased from New England Nuclear Dupont (Boston, MA, U.S.A.); fetal bovine serum (FBS) and α-minimum essential medium (α-MEM) were from Irvine Scientific (Santa Ana, CA, U.S.A.); the Ca-ionophore A23187 was from Calbiochem-Behring Co. (La Jolla, CA, U.S.A.); disodium α-glycerophosphate (α-GP) was from Tokyo Kasei Co. (Tokyo, Japan); purified prostaglandin H synthase (PHS) from ram seminal vesicles was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, U.S.A.); and thin layer silica gel plates (60F$_{254}$; layer thickness 0.25 mm) were from Merck Co. (Darmstadt, Germany).

Cell culture. All cells were obtained from patients who gave their informed consent prior to the procedure. Details of osteoblast isolation from the periosteum have been described previously [13, 14]. Periosteal specimens from the ulna were obtained from a 20 year-old patient undergoing surgery for treatment of traumatic fracture. In brief, cell populations that migrated from an explant were obtained by screening for alkaline phosphatase (ALP) activity above 160 IU/well. These cells (SaM-1) were cultured in α-MEM supplemented with 10% FBS at 37° in 5% CO_2/95% air. Confluent cells were dispersed with 0.05% trypsin and 0.05% EDTA and then transferred to new plastic dishes in a split ratio of 1:2 or 1:4. The culture medium was replaced three times each week. The cells were identified as osteoblasts according to the characteristic features displayed. Another osteoblast-like periosteal cell strain (IWF-2) was isolated from a femur periosteal specimen obtained from a 55 year-old patient who was also undergoing surgery for traumatic fracture. The skin fibroblasts, TF-S, had migrated from skin explants obtained from an 80 year-old patient. Fetal lung fibroblasts, TIG-2, were established in culture at our institute [15]. HSMC-3, aortic smooth muscle cells, were isolated from a newborn baby. MC3T3-E1, a clonal osteogenic cell line established from fetal mouse calvaria, is frequently used for basic research into bone metabolism [16]. All cell strains were cultured in α-MEM containing 10% FBS.

Incubation with menatetrenone. The effects of menatetrenone on cell growth were prevented by using confluent cells which had ceased growing. Cells at 18 population doubling levels, which had reached confluence, were incubated with menatetrenone dissolved in dimethyl sulfoxide (DMSO) in the presence of 2 mM α-GP as 10 mM β-GP, which accelerated the mineralization process [17]. The same amount of vehicle was added to control cultures. The final concentration of DMSO was less than 0.1%, and media were changed every other day. The cells were cultured for 5 days, or for the indicated number of days, before assay.

PG release into the medium. The amount of PG released into the medium by confluent cells incubated with menatetrenone on 24-multiwell dishes for 24 hr was determined after the medium had been replaced by serum-free medium for diploid cells, ASF-301 (Ajinomoto Co., Tokyo, Japan), to avoid the contamination of bovine PG in the FBS. Medium PG levels, i.e., those of PGE$_2$ and 6-keto-PGF$_{1\alpha}$, were directly assayed by RIA in accordance with the manufacturer's instructions.

Assay of PGH synthesizing activity in cell homogenates. Cells (4–5 × 10^6)/56 cm^2 incubated with or without various concentrations of menatetrenone in the presence of 2 mM α-GP for 5 days or for the indicated periods, were washed twice with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline [PBS(−)] and once with 0.05 M KH_2PO_4, pH 8.2. Cells (4–5 × 10^6) scraped in 1 mL phosphate buffer were homogenized by sonication. The assay for PGH synthesizing activity was carried out as reported previously [18, 19]. In brief, the resulting cell homogenate was incubated with 0.2 μCi [^{14}C]AA at 37° for 20 min, with shaking in an open tube. The reaction was terminated by adjusting the mixture to pH 3.0 with HCl, and the synthesized PG was extracted with 6 volumes of ethyl acetate. After evaporation, the residue was applied quantitatively to a thin layer chromatography plate and compared with PGD$_2$, E$_2$, F$_{2\alpha}$, thromboxane B$_2$, and 6-keto-PGF$_{1\alpha}$ as authentic standards, using the upper phase of solvent C, consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, v/v). Labeled PG separated on the plate were visualized by autoradiography. The radioactive zones were each scraped off the plate and the radioactivity was measured with a liquid scintillation spectrometer. In some experiments, indomethacin (5 × 10^{-4} M) was added to the assay to inhibit PHS. The PG synthesizing activity of the cells was expressed as the dpm of ^{14}C-labeled PG per mg protein of cell homogenate.

To investigate the direct effect of menatetrenone on PG synthesizing activity, untreated confluent cells (3–5 × 10^6), scraped and suspended in the phosphate buffer (1 mL), were sonicated. The resulting homogenates were incubated with various concentrations of menatetrenone and [^{14}C]AA, and synthesized [^{14}C]PG were separated as described above.

Assay of purified PHS activity. The purity of the PHS obtained from ram seminal vesicle, assayed by SDS–polyacrylamide gel electrophoresis, was 95% and it consisted of about 85% holoenzyme. The specific activity was 106,000 units/mg protein, one unit being the amount of enzyme that exhibited an initial velocity of one nanomole of O_2 per minute in conjunction with the conversion of AA to PGG$_2$ at 37°. Purified PHS (167 units) was incubated with assay buffer containing 0.1 M Tris–HCl (pH 8.0), 1 μM hematin, 5 mM L-tryptophan and 0.1 μCi [^{14}C]-AA at 37° for 10 min according to the method of Miyamoto *et al.* [20], with slight modifications. The assay was terminated by the addition of 0.45 mL of a mixture of ethylether/methanol/0.2 M citric acid (30/4/1). After the aqueous portion was removed with anhydrous sodium sulfate, the organic phase containing the synthesized [^{14}C]PG was spotted onto thin layer chromatography (TLC) plates and developed with the upper portion of solvent C at room temperature. The activity was expressed as the total radioactivity of the synthesized [^{14}C]PGs. Indomethacin (5 × 10^{-4} M) inhibited the activity by 99.5%.

Release of AA. To examine the effect of

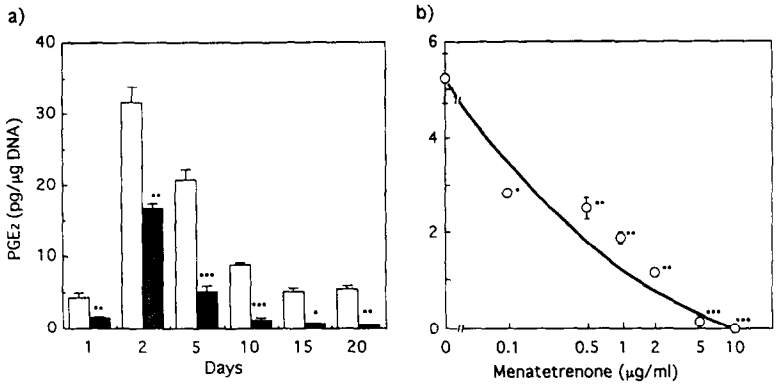

Fig. 1. Effects of menatetrenone on PGE_2 production in human osteoblast-like periosteal cells. (a) Confluent cells were incubated with (▨) or without (□) menatetrenone (1 μg/mL) for various periods. PGE_2 released into the medium over 24 hr was determined by RIA. (b) Confluent cells were incubated with various concentrations of menatetrenone for 5 days. PGE_2 released into the medium for 24 hr was determined by RIA. Each value indicates the mean ± SE (N = 4). Significant differences from values for untreated cells. *P < 0.05, **P, 0.01 and ***P < 0.001.

menatetrenone on the release of AA from osteoblast-like cells, a 4-cm^2 monolayer was incubated with 0.05 μCi [^{14}C]AA in α-MEM (containing 10% FBS) overnight. The culture medium was removed by two washes with α-MEM (containing 0.1% FBS) and the cells were exposed to various concentrations of menatetrenone and 10 μM A23187 to stimulate AA release in the α-MEM supplemented with 0.1% FBS. Radioactivity released into the medium was quantified by liquid scintillation measurement.

H_2O_2 *measurement.* To measure H_2O_2 formation after the addition of menatetrenone, monolayers of confluent cells were rinsed and incubated for 15 min with 2 mL of Hank's balanced salt solution containing menatetrenone. After exposure, the buffer was removed and assayed for H_2O_2 content, using the colorimetric assay described by Thurman *et al.* [21].

RESULTS

Effects of menatetrenone on PG production by osteoblast-like periosteal cells during mineralization

Human osteoblast-like periosteal cells produced significant amounts of PGE_2 and 6-keto-$PGF_{1\alpha}$, the latter being a stable metabolite of PGI_2, in the culture medium after 24 hr, as determined by RIA. After the confluent cells were treated with menatetrenone (1 μg/mL = 2.25×10^{-6} M) for various periods in the presence of α-GP, PGE_2 released into the medium 24 hr later was determined. Menatetrenone inhibited PGE_2 production in a time-dependent manner (Fig. 1a). PGE_2 production in untreated control cells was also reduced over time in culture in the presence of α-GP. Since the cells were exposed after reaching confluence, the cell number did not change. On day 1, menatetrenone significantly inhibited PG production, but the levels at this time had originally been lower than on other days. The reason for this result is unclear, but it might depend on an α-GP effect. The reduction was recovered after the cells were cultured for one more day. On day 2, the PG level in menatetrenone-treated cells had decreased to 50% that of untreated control cells. The inhibition ratio increased up to 85% over time in culture. Since menatetrenone inhibited PG production by 76% on day 5, the effects of various concentrations of menatetrenone on PGE_2 production were investigated in cells exposed to the agent for 5 days. Figure 1b shows that menatetrenone, at doses of 0.1–10 μg/mL, inhibited PGE_2 production in a dose-dependent manner, and that PGE_2 production was completely inhibited by menatetrenone (10 μg/mL) without any evidence of cytotoxicity (the DNA content of untreated and treated cells was the same). The half inhibition dose of menatetrenone (ID_{50}) was around 2.93×10^{-7} M. The production of 6-keto-$PGF_{1\alpha}$ was also inhibited (data not shown).

PG synthesizing activity in menatetrenone-treated cells

To determine whether menatetrenone influenced PG synthesizing activity, cells exposed to menatetrenone (1 μg/mL) for various periods were homogenized and incubated with exogenous [^{14}C]-AA. The PG synthesizing activity, determined as the total radioactivity of synthesized [^{14}C]PG, was suppressed by menatetrenone in a time-dependent manner (Fig. 2a). The activity was completely inhibited on day 1; however, at earlier time points, i.e. 3 and 6 hr, the activity was inhibited by only 14 and 23%, respectively (data not shown). The PG synthesizing activity in untreated cells was not reduced over time in culture. PG synthesizing activity was dose dependently inhibited in cells incubated with various concentrations of menatetrenone for 5 days. The ID_{50} was 1.35×10^{-6} M; this was 3-fold higher than that of PG production in intact cells treated with menatetrenone (Fig. 2b). The synthesis of both PGE_2 and 6-keto-$PGF_{1\alpha}$ was inhibited to the same degree as in untreated control cells (Fig. 3). This suggested that menatetrenone inhibits PHS activity, but not that of PGI_2 synthase on PGE_2 isomerase, since the same inhibition ratio of PGI_2

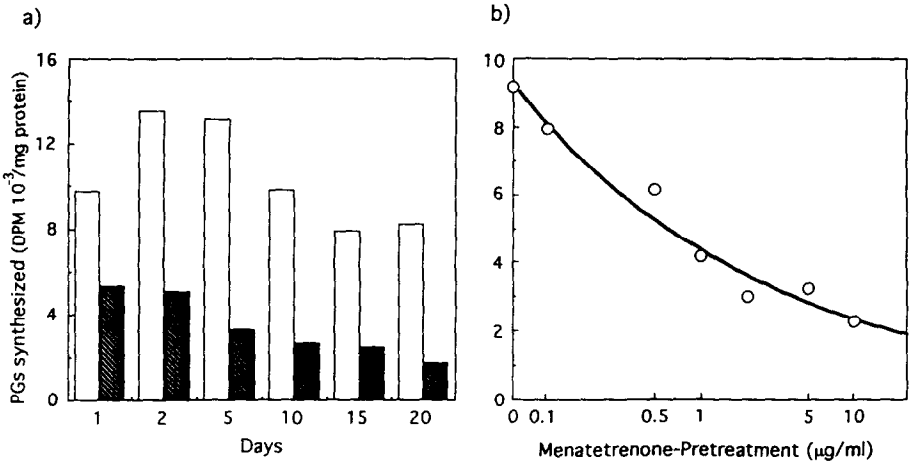

Fig. 2. Effects of menatetrenone pretreatment on PG synthesizing activity. (a) Confluent cells pretreated with (▨) or without (□) menatetrenone (1 μg/mL) for various periods were homogenized, then incubated with [^{14}C]AA. PG synthesizing activity is expressed as the total radioactivity of the synthesized [^{14}C]PGs. (b) Confluent cells incubated with various concentrations of menatetrenone for 5 days were assayed for PG synthesizing activity as described above. Indomethacin (5×10^{-4} M) inhibited the activity by 98%. The PG synthesized in the cell homogenate were mainly PGE_2 and 6-keto-$PGF_{1\alpha}$. Values indicate the means of duplicate assays. Reproducible results were obtained.

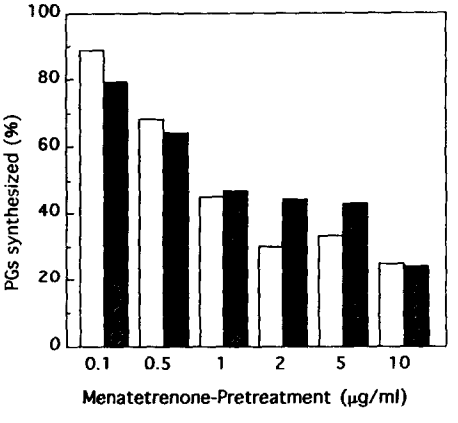

Fig. 3. Menatetrenone-pretreatment inhibits the synthesis of both PGE_2 and 6-keto-$PGF_{1\alpha}$. Amount of [^{14}C]PGE_2 and [^{14}C]6-keto-$PGF_{1\alpha}$ synthesized in the menatetrenone-pretreated cell homogenate as described in Fig. 2b are shown as percentages of the amount in the untreated control cell homogenate. (□) PGE_2 and (▨) 6-keto-$PGF_{1\alpha}$.

and PGE_2 synthesis from PGH_2 is unlikely as these enzymes have different sensitivities for hydrogen peroxide and other compounds [22].

To investigate the participation of newly-synthesized protein in the inhibition, confluent cells were incubated with menatetrenone (1 μg/mL) in the presence of cycloheximide (0.1 or 0.5 μg/mL) for 2 days. Cycloheximide inhibited protein synthesis by incorporating [^{3}H]proline into acid-insoluble materials to an extent of 71 and 92% at 0.1 and 0.5 μg/mL, respectively, concentrations at which it slightly inhibited PG synthesizing activity in cell homogenates. The menatetrenone-induced inhibition of PG synthesizing activity was recovered at both concentrations of cycloheximide (Table 1), this finding suggesting that menatetrenone induced some proteins that inhibited PG synthesizing activity.

Effects of menatetrenone on PG synthesizing activity in cell homogenates

Various concentrations of menatetrenone were added to untreated cell homogenates, and PG synthesizing activity was determined as described in Materials and Methods. Concentrations as high as 10 μg/mL inhibited PG synthesizing activity in the cell-free system (Fig. 4). However, at concentrations of 1 μg/mL, menatetrenone did not inhibit this activity. PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis was reduced to exactly the same extent by menatetrenone (data not shown). These results implied that menatetrenone inhibited PHS activity directly, but did not thus inhibit PGI_2 synthase and PGE_2 isomerase.

To investigate whether menatetrenone directly inhibited PHS activity, purified PHS from a ram seminal vesicle was incubated with various concentrations of the agent. PHS activity was determined as the total radioactivity of the non-enzymatic metabolites of PGH_2, [^{14}C]PGD_2, E_2 and $F_{2\alpha}$ synthesized from [^{14}C]AA. As shown in Fig. 5, menatetrenone at concentrations of greater than 1 μg/mL significantly inhibited PHS activity; this effect was more potent than that in the cell homogenate.

Effects of menatetrenone on phospholipase activity

Phospholipase activity was determined by measuring the A23187-induced release of radioactivity

Table 1. Effects of cycloheximide on menatetrenone-induced inhibition of PG synthesizing activity

Cycloheximide	PG synthesized (dpm × 10^{-3}/mg protein) Control	Menatetrenone	% of control
None added	4.42 ± 0.39	2.92 ± 0.20*	66
0.1 μg/mL	3.77 ± 0.28	3.75 ± 0.18	99
0.5 μg/mL	3.76 ± 0.46	4.41 ± 0.66	117

Confluent cells were incubated with menatetrenone (1 μg/mL) in the presence or absence of cycloheximide (0.1 or 0.5 μg/mL) for 2 days. PG synthesizing activity in the cell homogenate was assayed with [^{14}C]AA, as described in Materials and Methods.

Values are means ± SEM (N = 3). The same experiment was carried out twice and reproducible results were obtained. Significant difference compared with control. *$P < 0.05$.

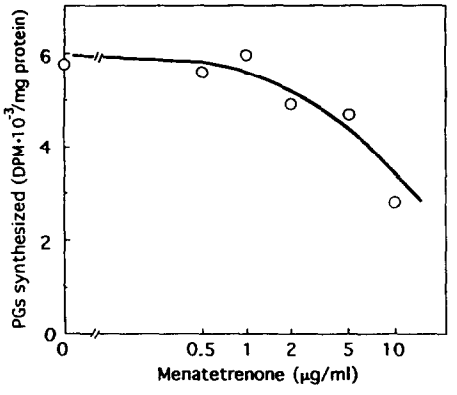

Fig. 4. Direct effects of menatetrenone on PG synthesizing activity in the cell homogenate. Untreated cell homogenate was incubated with various concentrations of menatetrenone and [^{14}C]AA at 37° for 20 min. PG synthesizing activity was assayed as described in Materials and Methods. The values indicate the means of duplicate assays. Reproducible results were obtained.

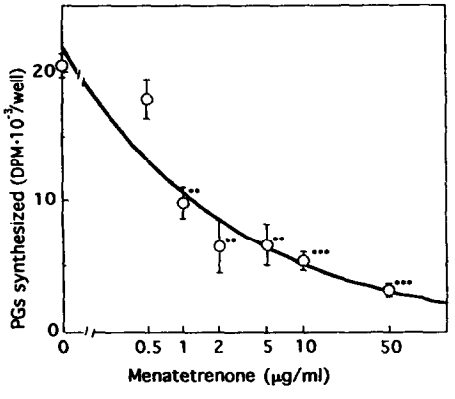

Fig. 5. Menatetrenone inhibits purified PHS activity. The activity of purified PHS (167 units) from a ram seminal vesicle was assayed in the presence of various concentrations of menatetrenone and [^{14}C]AA, as described in Materials and Methods. Values indicate the means ± SE (N = 3). The ID_{50} value of menatetrenone was 2.25×10^{-6} M.

from cells prelabeled with [^{14}C]AA. The A23187-induced release of [^{14}C]AA increased in a time-dependent manner, until 60 min. Menatetrenone (1 μg/mL) time dependently inhibited AA release (Fig. 6a), and also caused a dose-dependent inhibition in A23187-induced [^{14}C]AA release from the cell membrane (Fig. 6b). The maximum inhibition was 40%, at a concentration of 5 μg/mL. However, concentrations above 10 μg/mL did not exhibit any greater inhibition than those below 10 μg/mL. This might be due to the increased basal release of [^{14}C]AA caused by menatetrenone (10 μg/mL) in the absence of A23187. AA release induced by 10% FBS was slightly affected by menatetrenone.

Effects of menatetrenone on PG production in various cell types

In view of the above results in human osteoblast-like periosteal cells, we investigated the effects of menatetrenone on PG production in various other cell types to determine the cell specificity of this response. After they had reached confluence, various cell strains were incubated with different concentrations of menatetrenone (0.01–10 μg/mL) for 5 days, and the PGE_2 released into the medium was measured by RIA. PG production per μg DNA was inhibited by menatetrenone (1 μg/mL) in osteoblast-like periosteal cells and fibroblasts, but not in aortic smooth muscle cells and MC3T3-E1 cells (Table 2). All cell types used here, except MC3T3-E1, were normal human diploid cells with 46 chromosomes. Aortic smooth muscle cells of human diploid cell strains had slightly reduced cell growth and PG production in the presence of menatetrenone, whereas PGE_2 and 6-keto-$PGF_{1\alpha}$ production per cell was not affected. Sensitivity to menatetrenone differed depending on cell types. The ID_{50} ranged from 9.0×10^{-9} to 1.44×10^{-6} M, as shown in Table 2.

DISCUSSION

PG production in human osteoblast-like cells treated with menatetrenone was inhibited by a sublethal concentration of 2.25×10^{-6} M. PG production is inhibited in bovine aortic endothelial cells by phylloquinone (vitamin K_1), via the inhibition of phospholipase [10], and in porcine endothelial cells by menadione (vitamin K_3), via the inhibition

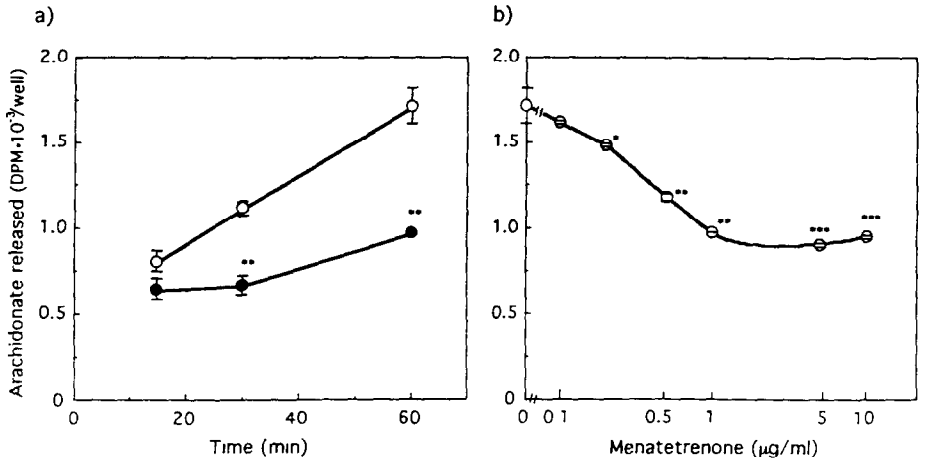

Fig. 6. Effects of menatetrenone on phospholipase activity. (a) [^{14}C]AA-labeled cells were incubated with (●—●) or without (○—○) menatetrenone (1 μg/mL) for various periods in the presence of 10 μM A23187. (b) [^{14}C]AA-labeled cells were incubated with various concentrations of menatetrenone for 60 min in the presence of 10 μM A23187. The [^{14}C]AA released into the medium was measured.

Table 2. Effects of menatetrenone on PGE_2 production in various cell strains

Cell strain	Phenotype	ID_{50} (μM)	PGE_2 production by menatetrenone* (dpm/μg DNA) without	with	% inhibition†
SaM-1	Osteoblasts	0.293	5.25 ± 0.52	1.87 ± 0.12§	64
IWF-2	Osteoblasts	0.009	1.59 ± 0.06	0.21 ± 0.01‖	87
MC3T3-E1	Osteoblasts (mouse)	—	5.77 ± 0.45	5.02 ± 0.52	13
TF-S	Skin fibroblasts	1.44	3.74 ± 0.21‡	1 64 ± 0.10‡‖	56
TIG-2	Lung fibroblasts	1.35	12.13 ± 1.29	4.52 ± 0.36§	63
HSMC-3	Aortic smooth muscle cells	—	0.96 ± 0.02	0.92 ± 0.25	5

Confluent cells were incubated with or without various concentrations (0.01–10 μg/mL) of menatetrenone for 5 days. PGE_2 released into medium was determined by RIA.

* Concentration of menatetrenone, 1 μg/mL.

† % inhibition indicates PGE_2 levels in treated cells relative to those in untreated cells.

‡ 13 days treatment.

Significant difference compared with untreated cells.

§ $P < 0.01$, ‖ $P < 0.001$. Values are means ± SEM (N = 3 or 4). Reproducible results were obtained at least twice.

of PGI synthase due to the increased intracellular production of H_2O_2 [11]. That is, the carboxylation of specific glutamyl residues to form gamma-carboxylates causes epoxidation of vitamin K to its 2,3-epoxide [23] and a hydroperoxide intermediate of vitamin K is thus involved in the carboxylation reaction. PHS, the rate limiting step for the conversion of AA to PG, is reportedly activated by low levels of exogenous hydroperoxides, whereas a high enough concentration of hydroperoxide causes autoinactivation [22, 24]. We cannot exclude the possibility that hydroperoxide was produced during intracellular menatetrenone metabolism, although we did not detect H_2O_2 formation either in cells incubated with or in those incubated without menatetrenone (data not shown). Menatetrenone, at 10 μg/mL, directly inhibited PG synthesizing activity in a cell-free system. In contrast, the incubation of ram seminal vesicle microsomes with 10^{-4} M phylloquinone and 2×10^{-5} M menadione had no direct effect on the conversion of AA. This suggested that menatetrenone inhibited PG production in a manner different from that of other vitamin K analogs. Although PGI_2 synthase is more sensitive to hydroperoxide than PHS, menatetrenone inhibited the conversion of AA to PGE_2 and 6-keto-

$PGF_{1\alpha}$ to the same extent (Fig. 3). This implied that menatetrenone inhibited PHS activity directly. We further confirmed that menatetrenone inhibited purified PHS activity (Fig. 5). However, the direct effect of menatetrenone on PHS activity after the addition to cell homogenates was weaker than the indirect effect of menatetrenone-pretreated cells on PHS activity. It is possible that menatetrenone might cause the production of down-regulation factors. Since menatetrenone-induced inhibition was reduced by cycloheximide, it is probable that newly-synthesized protein participates in this inhibitory effect. Further, the inhibitory activity induced by menatetrenone showed time-lag, that is, the PHS activity was inhibited 14, 23 and 38% at 3, 6 and 24 hr after menatetrenone treatment, respectively (data not shown).

Apart from the conversion of AA to PG, another important step in PG production is the release of AA from cell membrane phospholipid. The A23187-induced AA release in bovine endothelial cells was inhibited by 30-min pretreatment with phylloquinone [10], but in porcine endothelial cells it was not inhibited by 15-min menadione pretreatment [11]. Here we showed that menatetrenone inhibited the A23187-induced AA release in human osteoblast-like periosteal cells. However, the 10% serum-induced basal level of AA release was only slightly inhibited by menatetrenone, indicating that the inhibition of AA release might not be the main inhibitory effect of menatetrenone on PG production.

Menatetrenone, up to a concentration of 10 μg/mL, was not toxic towards the cell membrane as evidenced by the DNA content in the culture dishes remaining unchanged. Moreover, menatetrenone (0.01–1.0 μg/mL) showed no toxic acute effects, as determined by the investigation of [^{3}H]thymidine incorporation into DNA fraction in log phase cells. Our results showed that the inhibitory effects of menatetrenone on PG production in osteoblast-like periosteal cells were primarily due to the inhibition of phospholipase and PHS, and partly to the effect of newly-synthesized protein(s); however, which of these protein(s) were menatetrenone-induced is not known.

In untreated osteoblast-like periosteal cells during culture at the stationary phase, PGE_2 production was reduced (Fig. 1). These cells produced more PGE_2 in the growing than that in the stationary phase. This reduction in PGE_2 production is important in bone formation, since PGE_2 is the most potent bone resorbing factor [9]; PGI_2, another major PG produced in fetal rat long bone, has almost the same potency for bone resorption as PGE_2 [25]. Menatetrenone has been shown to inhibit PGE_2-induced bone resorption in mouse calvaria organ culture [8]. Moreover, when menatetrenone inhibited interleukin-1α-induced bone resorption, it reduced PGE_2 production in a dose-dependent manner.

The therapeutic effect of menatetrenone on involutional osteoporosis has recently been demonstrated by Orimo *et al.* [16]. Taken together, these findings indicate that menatetrenone may increase bone mass. Our findings suggest that this occurs via the inhibition of bone resorption. This, in turn, being due to the inhibition of PG production in osteoblasts.

REFERENCES

1. Olson RE, The function and metabolism of vitamin K. *Annu Rev Nutr* **4**: 281–337, 1984.
2. Suttie JW, Vitamin K-dependent carboxylase *Annu Rev Biochem* **54**: 459–477, 1985.
3. Hauscka PV, Lian JB and Gallop PM, Direct identification of the calcium-binding amino acid, gamma-carboxyglutamate, in mineralized tissue. *Proc Natl Acad Sci USA* **72**: 3925–3929, 1975.
4. Price PA, Otsuka AS, Poser JW, Kristaponis J and Raman N, Characterization of a γ-carboxyglutamic acid-containing protein from bone. *Proc Natl Acad Sci USA* **73**: 1447–1451, 1976.
5. Hauschka PV, Osteocalcin: the vitamin K-dependent Ca^{2+} binding protein of bone matrix. *Haemostasis* **16**: 258–272, 1986.
6. Bouckaert JH, Fracture healing vitamin K. *Nature* **19**: 849, 1960.
7. Hart JP, Catterall A, Dodds RA, Klenerman L, Shearer MJ, Bitensky L and Chayen J, Circulating vitamin K_1 levels in fractured neck of femur. *Lancet* **2**: 283, 1984.
8. Hara K, Akiyama Y, Taijima T and Shiraki M, Menatetrenone inhibits bone resorption partly through the inhibition of PGE_2 synthesis. *J Bone Miner Res* **8**: 535–542, 1993.
9. Raisz LG and Martin TT, Prostaglandins in bone and mineral metabolism. In: *Bone and Mineral Research, Annual 2* (Ed. Peck WA), pp. 286–310. Elsevier, New York, 1984.
10. Nolan RD and Eling TE, Inhibition of prostacyclin synthesis in cultured bovine aortic endothelial cells by vitamin K_1. *Biochem Pharmacol* **35**: 4273–4281, 1986.
11. Barchowsky A, Tabrizi K, Kent RS and Whorton AR, Inhibition of prostaglandin synthesis after metabolism of menadione by cultured porcine endothelial cells. *J Clin Invest* **83**: 1153–1159, 1989.
12. Larson AE and Suttie JW, Vitamin K-dependent carboxylase: evidence for a hydroperoxide intermediate in the reaction. *Proc Natl Acad Sci USA* **75**: 5413–5416, 1978.
13. Koshihara Y, Kawamura M, Oda H and Higaki S, *In vitro* calcification in human osteoblastic cell line derived from periosteum. *Biochem Biophys Res Commun* **145**: 651–657, 1987.
14. Koshihara Y, Kawamura M, Endo S, Tsutsumi C, Kodama H, Oda H and Higaki S, Establishment of human osteoblastic cells derived from periosteum in culture. *In Vitro Cell Dev Biol* **25**: 37–43, 1989.
15. Ohashi M, Aizawa S, Ooka H, Ohsawa T, Kaji K, Kondo H, Kobayashi T, Noumura T, Matsuo M, Mitsui Y, Murota S, Yamamoto K, Ito H, Shimada H and Utakoji T, A new human diploid cell strain, TIG-1, for the research on cellular aging. *Exp Gerontol* **15**: 121–133, 1980.
16. Sudo H, Kodama HA, Amagai Y, Yamamoto S and Kasai S, *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* **96**: 191–198, 1983.
17. Ecarot-Charrier B, Glorieux FH, van Der Rest M and Pereira G, Osteoblast isolation from mouse calvaria initiates matrix mineralization in culture. *J Cell Biol* **96**: 639–643, 1983.
18. Murota S, Kawamura M and Morita I, Transformation of arachidonic acid into thromboxane B_2 by the homogenates of activated macrophages. *Biochim Biophys Acta* **528**: 507–511, 1978.
19. Koshihara Y, Inagaki A and Murota S, Induction of

prostacycline formation by sodium *n*-butyrate in a cloned epithelial cell line. *Biochim Biophys Acta* **664**: 278–290, 1981.
20. Miyamoto T, Yamamoto S and Hayaishi O, Prostaglandin synthetase system-resolution into oxygenase and isomerase components. *Proc Natl Acad Sci USA* **71**: 3645–3648, 1974.
21. Thurman RG, Ley HG and Scholz R, Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. *Eur J Biochem* **25**: 420–430, 1972.
22. Kulmacz RJ, Prostaglandin H synthase and hydroperoxides: peroxidase reaction and inactivation. *Arch Biochem Biophys* **249**: 273–285, 1986.
23. Sadowski JA, Schnoes HK and Suttie JW, Vitamin K epoxidase: properties and relationship to prothrombin synthesis. *Biochemistry* **16**: 3856–3863, 1977.
24. Marshall PJ, Kulmacz RJ and Lands WEM, Constraints on prostaglandin biosynthesis in tissue. *J Biol Chem* **262**: 3510–3517, 1987.
25. Raisz LG, Vanderhoek JY, Simmons HA, Kream BE and Nicholaou KC, Prostaglandin synthesis by fetal rat bone *in vitro*: evidence for a role of prostacyclin. *Prostaglandin* **17**: 905–914, 1979.
26. Orimo H, Fujita T, Onomura T, Inoue T, Kushida K and Shiraki M, *Clin Evol* (abstract in English) **20**: 45–100, 1992.