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BARBITURATES ENHANCE RETINOIC ACID OR 1,25-DIHYDROXYVITAMIN D_3-INDUCED DIFFERENTIATION OF LEUKEMIA HL-60 CELLS

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SUMMARY: In the presence of 1 nM retinoic acid (RA), pentobarbital markedly enhanced differentiation of HL-60 cells to granulocytic cells. In the absence of RA, pentobarbital by itself did not induce cell differentiation. Similarly, pentobarbital enhanced the action of 1,25-dihydroxyvitamin D3 to induce differentiation of HL-60 cells into monocyte/macrophage lineage. The potency of various barbiturates to enhance cell differentiation was closely correlated with their activity to inhibit protein kinase C of HL-60 cells. In contrast to staurosporine, however, barbiturates did not affect the action of differentiation inducers of other types such as dimethyl sulfoxide, dibutyryl cyclic AMP or actinomycin D.

In spite of widespread use of barbital and its derivatives as sedatives or hypnotics, mechanism of their action still remains unclear. It is most probable that cell membrane is the site of their action (1). In this regard, suggestive are recent reports that phenobarbital is an effective inhibitor of protein kinase C (PKC) (2,3) and that PKC is playing an important role in the process of differentiation of various cells (4-6).

HL-60 cells which we used in the present study are human promyelocytic leukemia cells possessing a binary potential of differentiating either to granulocytic cells or to monocytic cells depending on chemical nature of inducers of differentiation. For example, all-trans-beta-retinoic acid (RA), actinomycin D (Act D) or dimethylsulfoxide (DMSO) make HL-60 cells to become granulocytic cells whereas 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) make them to differentiate into monocytic cells (7). The list of inducers of HL-60 cell differentiation includes inhibitors of protein kinase C (PKC) such as staurosporine (8).

The present study reports that barbiturates enhanced differentiation of HL-60 cells either to granulocytic lineage or to monocytic lineage in the presence of cell lineage-specific inducers, although barbiturates by themselves had no activity of inducing differentiation of the cells to either

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direction. It also reports a correlation between the differentiation-inducing action of barbiturates and their action of inhibiting PKC in HL-60 cells.

MATERIALS AND METHODS

Cell culture. HL-60 cells were supplied by Japan Cancer Research Bank and maintained in culture at 37°C in a humidified 5% CO_2 atmosphere or stored in liquid nitrogen. The cells were cultured in plastic petri dishes by the use of RPMI 1640 tissue culture medium (Gibco) supplemented with 10% fetal calf serum (Flow). Initial cell density was 500,000 cells/ml. The number of cells was determined by a hemocytometer while cell viability was measured by the ability of the cells to exclude trypan blue.

Assessment of differentiation of HL-60 cells. Ethanol was used to dissolve $1,25(OH)_2D_3$ (Kyowa Hakko Co., Tokyo) whereas RA (Sigma) was dissolved in DMSO and TPA in acetone. The final concentrations of these organic solvents were less than 0.05%, at which concentration they themselves did not affect the differentiation process. Except otherwise specified, barbiturates and differentiation inducers were given to the cells at the same time.

Reduction of nitroblue tetrazorium (NBT) and resulted intracellular deposit of blue-black formazan was used as an indicator of differentiated cells of both granulocytic and monocytic lineages according to the reported method (9). At least 800 cells were scored for each determination and the result was expressed in the percentage of NBT-positive cells in the population of viable cells. Fully differentiated cells were examined for their morphology after stained with the May-Grunwald Giemsa solution. The NBT method was adopted for routine assay, because cells at early stages of differentiation could be detected more easily by the NBT method than by the morphological method.

PKC assay. Activity of PKC of HL-60 cells was determined by the modification of the reported method (10). The cells were suspended in 20 mM Tris-Cl (pH 7.5) containing 10 mM EGTA, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 0.01% w/v leupeptin and 0.3% w/v beta-mercaptoethanol. The cells were lysed at 0°C by sonication and the cell debris was removed by centrifugation for 30 min at 100,000 x g. After appropriately diluted, the supernatant fluid was subjected to the PKC assay. The reaction mixture contained 20 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 μM [γ-^{32}P]ATP (10 μCi/ml), 75 μM oligopeptide EGF receptor 651-658 (Amersham), 40 μg/ml phosphatidyl serine, 1 μg/ml diacylglycerol, 2.5 mM dithiothreitol and 1 mM EGTA. The mixture (200 μl/tube) was incubated at 25°C for 15 min. The radiolabelled oligopeptide was precipitated by 10% trichloroacetic acid, collected on plastic filter membrane (Millipore) and subjected to radioactivity determination by a liquid-scintillation spectrometer.

RESULTS

Enhancement of granulocytic differentiation. Pentobarbital significantly increased NBT-positive HL-60 cells in the presence of 1 nM RA. Enhancement of cell differentiation occurred in a manner dependent on the concentration of pentobarbital and reached saturation at 0.8 mM (Fig. 1). Morphological examination of fully differentiated cells confirmed that granulocytes were produced by the combination of 0.8 mM pentobarbital and 1 nM RA.

A separate experiment showed that the number of NBT-positive cells in the presence of 1 nM RA and 0.8 mM pentobarbital increased linearly with time, reaching a plateau at 85% differentiation on Day 4 or 5. Pentobarbital by itself did not promote differentiation of the cells in the absence of RA

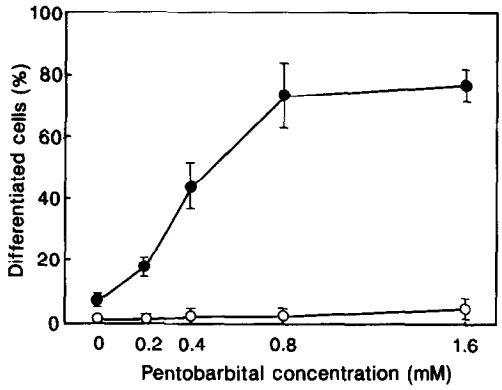

Figure 1. Effect of pentobarbital on differentiation of HL-60 cells in the presence (●) or absence (○) of 1 nM retinoic acid. The number of differentiated cells was determined on Day 4.

(Fig. 1) and RA alone at this low concentration was also practically powerless for the cell differentiation.

Removal of pentobarbital after contact with the cells for 1 or 3 days resulted in only 20 or 55% differentiation on Day 5, indicating that the incessant presence of pentobarbital was required for the maximal development of differentiation in the RA-treated cells. When RA was given to the cells which had been cultured with pentobarbital for the preceding 2 days, the number of differentiated cells started to increase linearly with time thereafter and reached 60% differentiation on Day 6. The cells thus remained undifferentiated in the presence of pentobarbital while they retained the capability to react to RA.

By 1 μM RA (i.e., 1000 times higher than the above), 43 ± 6, 62 ± 8 and 96 ± 3% of cells were driven to differentiate in 2, 3 and 5 days, while combination of 0.8 mM pentobarbital and 1 μM RA resulted in 72 ± 5, 96 ± 2 and 98 ± 2% differentiation in 2, 3 and 5 days. Thus, in addition to the above-mentioned mode of action (i.e., increase of final frequency of differentiated cells in the presence of 1 nM RA), pentobarbital showed another mode of action (i.e., acceleration of the velocity of differentiation in the presence of 1 μM RA).

Enhancement of monocytic differentiation. Differentiation of HL-60 cells to the cells of monocyte/macrophage lineage was also promoted by pentobarbital, when $1,25(OH)_2D_3$ was used at 1 nM as an inducer (Table I). Besides NBT reduction, the developed macrophage-like cells showed adhesiveness and phagocytic activity. At 1 nM, $1,25(OH)_2D_3$ by itself was not fully active to promote the cell differentiation. Other barbiturates such as phenobarbital or 2-thiobarbituric acid showed a similar differentiation-enhancing effect, although they were less powerful than pentobarbital. Barbital and barbituric acid were much less effective in this regard.

Table I
Enhancement by barbiturates of the action of retinoic acid or 1,25-dihydroxyvitamin D_3 to induce differentiation of HL-60 cells

Compounds[a]	Differentiation of HL-60 cells (% on Day 4)[b]	
	to granulocytic cells by 1 nM retinoic acid	to monocytic cells by 1 nM $1,25(OH)_2D_3$
Control	7 ± 3 (23)[c]	7 ± 4 (23)
Pentobarbital	74 ± 9* (20)	54 ± 5* (19)
Phenobarbital	45 ± 6* (21)	41 ± 6* (21)
2-Thiobarbituric acid	19 ± 3* (18)	11 ± 4 (18)
Barbital	11 ± 3 (20)	9 ± 1 (21)
Barbituric acid	9 ± 3 (18)	8 ± 4 (19)

[a] These compounds were used in a final concentration of 0.8 mM and given to the cells at the same time when they received either RA or $1,25-(OH)_2D_3$.
[b] Percentage of NBT-positive cells among viable cells was determined on Day 4. Values are the mean ± SD of 12 determinations (3 dishes, 4 samples/dish, 200 cells/sample). The asterisk means that the difference from the control is statistically significant at $P < 0.05$.
[c] Figures in the parentheses represent the number of viable cells (x 10^5/ml) at the end of culture.

Inhibition of protein kinase C. Addition of 1 mM pentobarbital to the solubilized PKC of HL-60 cells resulted in about 30% inhibition of the enzyme activity (Fig. 2). Pentobarbital in higher concentrations caused larger inhibition, and phenobarbital was nearly equivalent to pentobarbital in the potency to inhibit PKC. Both barbital and 2-thiobarbituric acid were much less inhibitory than either pentobarbital or phenobarbital, while barbituric acid was practically nontoxic to the enzyme.

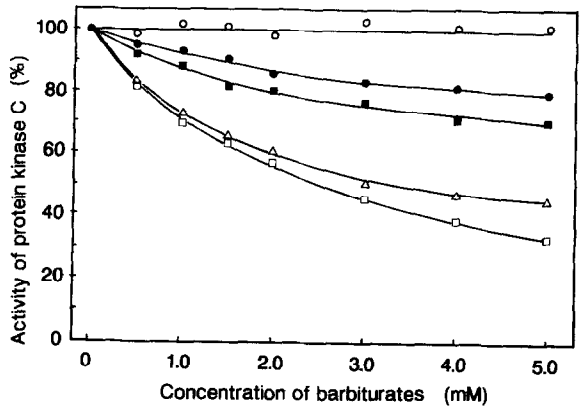

Figure 2. Effect of various barbiturates on the protein kinase C activity of HL-60 cells. The enzyme activity is expressed in % of control (i.e., incubation without any barbiturates; 25,870 ± 1210 cpm/tube). Each point represents the average of duplicated assays. Barbituric acid (○), sodium barbital (●), 2-thiobarbituric acid (■), phenobarbital (△), pentobarbital (□).

Table II
Effect of staurosporine and pentobarbital on the differentiation of HL-60 cells in the presence of dimethyl sulfoxide, dibutyryl cyclic AMP, actinomycin D or tetradecanoylphorbol acetate

Compounds	Differentiation of HL-60 cell (% on Day 4)[a]		
	Saline	Staurosporine (5 nM)	Pentobarbital (0.8 mM)
Control (saline)	1 ± 1 (31)[b]	2 ± 1 (24)	3 ± 2 (22)
Granulocyte inducers			
DMSO (100 mM)	3 ± 2 (22)	38 ± 6* (17)	3 ± 2 (12)
dbc AMP (106 μM)	0 ± 1 (22)	79 ± 3* (17)	0 ± 1 (20)
Act D (2.4 nM)	1 ± 1 (17)	35 ± 2* (13)	1 ± 1 (14)
Monocyte/macrophage inducer			
TPA[c] (1 nM)	5 ± 3 (25)	2 ± 2 (20)	3 ± 1 (19)
TPA (50 nM)	66 ± 5* (6)	2 ± 2 (12)	5 ± 2 (11)

[a] The percentage of NBT-positive cells among viable cells was determined on Day 4. The cells received either staurosporine or pentobarbital at the time of addition of inducer. In the presence of indicated concentration of staurosporine or pentobarbital, 50-80% of the cells were differentiated by 1 nM retinoic acid or 1 nM 1,25-dihydroxyvitamin D_3 (not shown in the table).

[b] Values are the mean ± SD (see the legend of Table I). Figures in the parentheses represent the number of viable cells (x 10^5/ml). * = significantly different ($P < 0.05$) from the controls.

[c] In the case of TPA, the percentage of differentiated cells was determined by the number of adherent cells on Day 2.

Difference between staurosporine and barbiturates. Pentobarbital had no enhancing effect on differentiation of HL-60 cells, when inducers other than RA or $1,25(OH)_2D_3$ were used as a granulocytic inducer in such low concentrations that differentiation was not induced by the inducer alone (Table II). Phenobarbital and three other barbiturates also failed to enhance the action of these inducers (data not shown). The result was in sharp contrast to that obtained by staurosporine (a potent PKC inhibitor); the action of all the three granulocytic inducers was significantly enhanced by staurosporine. On the other hand, pentobarbital mimicked staurosporine to inhibit monocytic differentiation of the cells in the presence of an optimal concentration of TPA.

DISCUSSION

It seems that the effect of pentobarbital to enhance HL-60 cell differentiation occurs specifically when RA or $1,25(OH)_2D_3$ is employed as the inducer. Both RA and $1,25(OH)_2D_3$ are known to act through binding to their specific nuclear receptors (11), although they drive HL-60 cells to different directions, i.e. RA to granulocytes whereas $1,25(OH)_2D_3$ to monocytes. Pentobarbital inhibited PKC of HL-60 cells and its analogues which were less inhibitory on PKC were less effective to enhance nuclear-receptor supported

cell-differentiation. The result suggests that release from suppressive effect of PKC intensifies the action of the nuclear receptors.

The difference between pentobarbital and staurosporine should be noted. Staurosporine is bound to the ATP-binding domain of PKC, while pentobarbital inhibits PKC probably through competition with diacylglycerol (3). Although pentobarbital is required in 10^5-fold higher concentrations than staurosporine for the enhancement of cell differentiation, the spectrum of its action seems to be narrower than that of staurosporine (Table II). In this regard, the action of pertussis toxin (PT) is as largely restricted as that of pentobarbital; PT inhibits RA but not DMSO in the process of granulocytic differentiation of HL-60 cells (12). On the other hand, PT differs from pentobarbital in that it does not affects the action of $1,25(OH)_2D_3$ which is susceptible to pentobarbital. In the present study, pentobarbital inhibited monocytic differentiation which was caused by 50 nM TPA. Also, pentobarbital did not enhance cell differentiation in the presence of suboptimal concentration of TPA. The result is comprehensible since TPA acts through activation of PKC which is susceptible to inhibition by pentobarbital.

In conclusion, the present study suggests that barbiturates are distinct from other agents in the specificity of differentiation-enhancing action and provide a useful tool for the study of regulation of nuclear receptors.

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REFERENCES

1. Seeman, P. (1972) Pharmacol. Rev. 24, 583-655.
2. Mikawa, K., Maekawa, N., Hoshina, H., Tanaka, O., Shirakawa, J., Goto, R., Obara, H. and Kusunoki, M. (1990) J. Int. Med. Res. 18, 153-160.
3. Chauhan, V.P.S. and Brockerhoff, H. (1987) Life Sci. 40, 89-93.
4. Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, I., Mikawa, K. and Nishizuka, Y. (1990) FEBS Lett. 263, 31-34.
5. Minana, M.-D., Felipo, V. and Grisolia, S. (1990) Proc. Soc. Natl. Acad. Sci. USA 87, 4335-4339.
6. Lacal, J.C., Cuadrado, A., Jones, J.E., Trotta, R., Burstein, D.E., Thomson, T. and Pellicer, A. (1990) Mol. Cell. Biol. 10, 2983-2990.
7. Collins, S.J. (1987) Blood 70, 1233-1244.
8. Okazaki, T., Kato, Y. Mochizuki, T., Tashima, M., Sawada, H. and Uchino, H. (1988) Exp. Hematol. 16, 42-48.
9. Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458-2462.
10. Fenerstein, N., Nishikawa, M. and Cooper, H.L. (1985) Cancer Res. 45, 3243-3251.
11. Evans, R.M. (1988) Science 240, 889-895.
12. Hemmi, H., Nakamura, T., Goto, Y. and Sugamura, K. (1989) Biochem. Biophys. Res. Comm. 163, 797-802.