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SPHINGOSINE INHIBITION OF TYROSINE AMINOTRANSFERASE AND TRYPTOPHAN OXYGENASE INDUCTION BY DEXAMETHASONE IN PRIMARY CULTURE OF RAT HEPATOCYTES

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SUMMARY: The effect of sphingosine, a known selective inhibitor of protein kinase C, on the induction of tyrosine aminotransferase (TAT) and tryptophan oxygenase (TO) by dexamethasone was studied in the primary culture of rat hepatocytes to determine the possible involvement of protein kinase C in the expression of glucocorticoid action. Sphingosine inhibits the induction of TAT by dexamethasone in a concentration-and time-dependent manner in primary culture of rat hepatocytes. It does not inhibit the induction of TAT by Bt_2cAMP. Sphingosine inhibits also the induction of TO by dexamethasone in a manner similar to TAT inhibition. It has no effect on the activity of lactate dehydrogenase, a cytosolic marker enzyme and on the protein content of the cultured hepatocytes. These findings indicate that endogenous modulator of protein kinase C, such as sphingosine, may influence the expression of glucocorticoid action in rat hepatocytes.

It has been considered that the hormone action on target cells are controlled not only by the concentration of hormones and their cognate receptors but also by modulators of these hormonal actions (1). Tyrosine aminotransferase (EC 2.6.1.5; TAT) and tryptophan 2,3-dioxygenase (EC 1.13.11.11; TO) are liver-specific enzymes, induced by various hormones, such as

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Abbreviations used: TAT, tyrosine aminotransferase; TO, tryptophan 2,3-dioxygenase; PKC, protein kinase C; LDH, lactate dehydrogenase; Bt_2cAMP, $N^{6,2'}$-O-dibutyryl-adenosine 3'5'-cyclic monophosphate.

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glucocorticoids, cAMP, glucagon and insulin (2-4) and have served as a model for the study of multihormonal regulation of gene expression. Glucocorticoids are Known to induce TAT and TO by activating transcription. They bind to intracellular receptor to form complexes which subsequently undergo activation and translocate to the nucleus where they interact with specific acceptor sites on chromatin and thus modulate gene expression (5). The role of receptor phosphorylation in DNA binding, trans-activation, and recycling of the receptor is not yet known, so as the involvement of endogenous kinase(s) and phosphatase(s) acting on the receptor (6,7). Previously, we have reported the involvement of PKC in glucocorticoid action (1).

Sphingosine and lysosphingolipids are potentially natural selective inhibitors of PKC and thus may serve to dissect the function of PKC in different cell systems (8). In the present paper, we report that sphingosine inhibits the induction of TAT and TO by dexamethasone in primary culture of rat hepatocytes.

MATERIALS AND METHODS

Materials: Wistar strain male rats of 150-200 g were used for preparation of hepatocytes. D-sphingosine, Bt_2cAMP, dexamethasone, methohemoglobin were from Sigma Chemical Co., USA. All other reagents were purest grade available commercially.

Primary culture of rat hepatocytes: Hepatocytes were isolated from adult male Wistar strain rats by *in situ* perfusion of the liver with collagenase (9). The cells were suspended at a density of 5×10^5 cells/ml in 6-cm collagen-coated dishes in Williams medium E containing 10% fetal calf serum, 10^{-6} M dexamethasone-phosphate, 10^{-7} M insulin, penicillin G (50 mg/l), Amphotericin B (0.25 mg/l) stryptomycin (130 mg/l) at 37°C under 5% CO_2 in air. After 48 hrs, the medium was replaced by hormone-free medium containing 10% fetal calf serum. One-day cultures were washed twice with serum free medium and used for the experiment.

Assay of biological activity: Dexamethasone and Bt_2cAMP were added to the cultures in serum-free medium as reported earlier (1). For the assay of TO activity, 2.5 mM tryptophan was added in serum-free medium. D-Sphingosine was dissolved in ethanol as stock solution of 50 mM and stored at -20°C. Before use, it was kept at 37°C water bath for few min and added to serum-free medium at desired concentrations. At indicated times, hepatocytes were washed twice with ice-cold phosphate buffered saline without divalent cations and harvested with a rubber policeman in 1.0 ml of homogenization buffer (0.25 M sucrose/50 mM potassium phosphate buffer pH 7.5/1 mM 2-oxoglutarate/48 µM pyridoxal phosphate) for the assay of TAT. Cells were sonicated for 5-7s at output control of 10 by Microson ultra-

sonic cell disruptor and centrifuged at 10,000 xg for 15 min at 2°C. The supernatant fractions were used for the assays of TAT and LDH. Hepatocytes were harvested and homogenized in 20 mM potassium phosphate buffer, pH 7.0 containing 2.5 mM tryptophan and 2 µM methohemoglobin and used for the assay of TO.

Enzyme and protein assays: The activities of TAT and TO were measured by the method of Granner and Tomkins (10) and Seglen and Jervell (11), respectively. LDH was assayed by the method of stolzenbach (12). The protein concentrations of the supernatant fractions used for the assays of TAT and LDH and of homogenate used for the assay of TO activity were determined by the method of smith et al. (13) and Bradfood (14), respectively. Enzyme activity is expressed as µmol of p-hydroxyphenyl pyruvate formed/min/mg protein for TAT, µmol of kynurenine formed/h/mg protein for TO and µmol of NADH oxidized/min/mg protein for LDH.

RESULTS AND DISCUSSION

Sphingosine and lysosphingolipids are potent and reversible natural inhibitors of PKC activity in vitro and in cell systems and that the inhibition of PKC requires the hydrophobic character and the positively charged amines (8,15). PKC, a Ca^{2+}-and phospholipid-dependent protein kinase, plays a central role in the transduction of extracellular signals into a cellular response (16). A possible involvement of PKC in mediation of glucocorticoid action expression has been viewed in this laboratory (1). In the present study, we used sphingosine, a selective inhibitor of PKC, to elucidate the involvement of PKC and sphingolipids in glucocorticoid regulation of TAT and TO induction.

Figure 1 shows that dexamethasone induces the specific activity of TAT by 4-fold and that the sphingosine inhibits significantly (35-40%) the dexamethasone mediated induction of TAT in primary culture of rat hepatocytes. Its inhibitory effect is in a concentration-and time-dependent manner (Fig. 2A & B). The maximum inhibitory effect is observed at 8 hr during which the dexamethasone inducibility of TAT activity reaches peak value (1). Sphingosine mediated inhibition of TAT induction by dexamethasone reaches plateau at 50 µM concentration. At this concentration of sphingosine, cell viability by trypan blue was over 95%. It was further confirmed by the measurement of LDH activity. The activity of LDH, a cytosolic marker enzyme, was same in all the experimental tests (Fig. 3) so as the protein content of the supernatant fractions (data

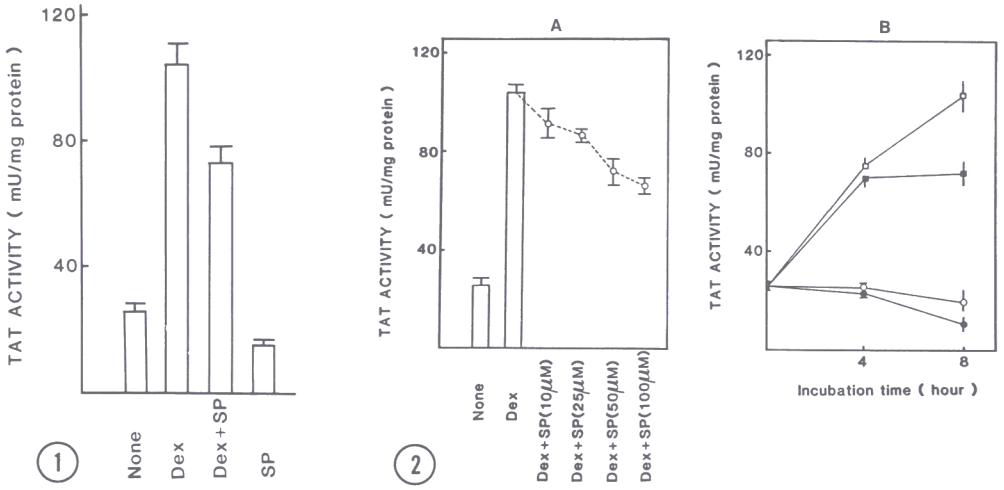

Fig. 1. Effect of sphingosine on the induction of TAT by dexamethasone in rat hepatocytes. The preparation of primary hepatocytes and incubation conditions are described in Materials and Methods. TAT activity of hepatocytes incubated with serum-free medium (None), 10^{-7} M dexamethasone (Dex), 10^{-7} M dexamethasone plus 50 μM sphingosine (Dex + SP) and 50 μM sphingosine alone (SP) for 8 hrs. Values are means from four dishes. Bars, S.D.

Fig. 2. Dose-response and time-course of sphingosine inhibition of TAT induction by dexamethasone in rat hepatocytes. A, TAT activity of hepatocytes incubated as those in Fig. 1 with indicated concentrations of sphingosine. B, TAT activity of hepatocytes incubated with serum-free medium with (●—●) or without (○—○) sphingosine (50 μM) and with 10^{-7} M dexamethasone with (■—■) or without (□—□) sphingosine (50 μM) for indicated times. Values are means form four dishes. Bars, S.D.

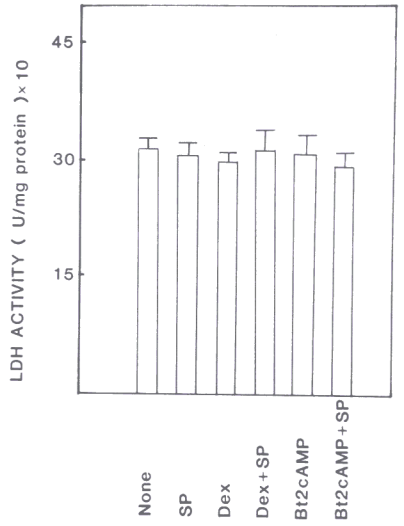

Fig. 3. Effects of sphingosine, dexamethasone and Bt_2cAMP on the LDH activity in cultured rat hepatocytes. Hepatocytes were treated same as in Fig. 1 & 4. LDH activity was measured as described in Materials and Methods. Values are means from 4-5 dishes. Bars, S.D.

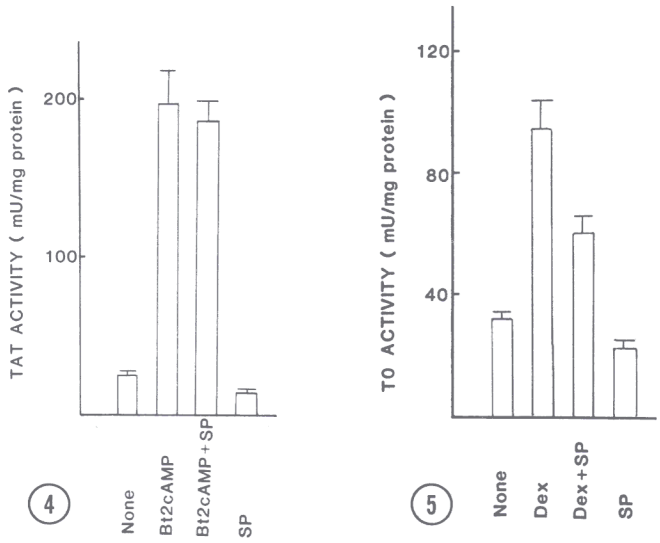

Fig. 4. Effect of sphingosine on the induction of TAT by Bt_2cAMP in rat hepatocytes. TAT activity of hepatocytes incubated with serum-free medium (None), 10^{-4} M Bt_2cAMP (Bt_2cAMP), 10^{-4} M Bt_2cAMP plus 50 μM sphingosine (Bt_2cAMP+SP) and sphingosine (SP) alone for 8 hrs. Experimental procedures are same as those of Fig. 1. Values are means from four dishes. Bars, S.D.

Fig. 5. Effect of sphingosine on the induction of TO by dexamethasone in rat hepatocytes. TO activity of hepatocytes incubated with serum-free medium (None), 10^{-6} M dexamethasone (Dex), 10^{-6} M dexamethasone plus 50 μM sphingosine (Dex + SP) and 50 μM sphingosine alone (SP) for 10 hrs. Values are means from four assays of four dishes each. Bars, S.D.

not shown). These data negate the possible cytotoxic effect of sphingosine in the present experiment.

In contrast, sphingosine does not inhibit significantly the Bt_2cAMP mediated induction of TAT (Fig. 4). This suggests its specificity towards glucocorticoid action. We also observed that sphingosine alone inhibits the activity of TAT, indicating that even basal level of TAT activity may be regulated by sphingosine. In an attempt to know whether sphingosine effect on TAT induction by dexamethasone is a general phenomenon, we also studied the effect of sphingosine on the induction of TO. Our data (Fig. 5) show that dexamethasone induces (3-fold) the specific activity of TO and sphingosine inhibits the induction of TO by dexamethasone in a manner similar to the inhibition of TAT. These findings indicate that sphingosine, an endogenous modulator of PKC, may influence the expression of glucocorticoid action.

Molecular mechanism for the regulatory site of PKC in glucocorticoid action is not yet known. A physiological role of

phosphorylation and dephosphorylation in glucocorticoid receptor function is still awaited. Although, glucocorticoid receptor is a phosphoprotein, the function of phosphorylation/ dephosphorylation, kinase(s) and phosphatase(s) acting on receptor is still under investigation (7, and ref. therein). Presently it is hard to attribute the direct functional site for PKC to glucocorticoid receptor for such a physiological phenomenon of PKC dependent regulation of glucocorticoid action. Albeit, the possibility of phosphorylation/dephosphorylation of some trans/cis acting factors involved in glucocorticoid regulation of gene expression cannot be ruled out (6,17).

Our findings suggest that sphingosine may be one of the endogenous factors which through PKC or some unidentified mechanism(s) regulate the TAT and TO activities expression by glucocorticoids either at the receptor or at the post receptor level. Effect of sphingosine on glucocorticoid receptor phosphorylation and its role in receptor function is of immediate interest.

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REFERENCES

1. Katunuma, N., Kato, Y., and Kido, H. (1988) Adv. Enzyme Reg. 27, 277-286.
2. Granner, D.K., and Hargrove, J.C. (1983) Mol. Cell. Biochem. 54, 113-128.
3. Greengard, O. (1971) Essays Biochem. 7, 159-205.
4. Nakamura, T., Niimi, S., Nawa, K., Noda, C., Ichihara, A., Takagi, Y., Arai, M., and Sakaki, Y. (1987) J. Biol. chem. 262, 727-733.
5. Yamamoto, K.R. (1985) Annu. Rev. Genet. 19, 209-215.
6. Beato, M. (1989) Cell 56, 335-344.
7. Auricchio, F. (1989) J. Steroid Biochem. 32, 613-622.
8. Hannun, Y.A., and Bell, R.M. (1989) Science 243, 500-507.
9. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) J. Biochem. (Tokyo) 84, 937-946.
10. Granner, D.K., and Tomkins, G.M. (1970) Methods in Enzymol. 17A, 633-637.
11. Seglen, P.O., and Jervell, K.F. (1969) Biochim. Biophys. Acta 171, 47-57.

12. Stolzenbach, F. (1966) Methods in Enzymol. 9, 278-288.
13. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Anal. Biochem. 150, 76-85.
14. Bradfood, M. (1976) anal. Biochem. 72, 248-254.
15. Merrill Jr., A.H., and Stevens, V.L. (1989) Biochim. Biophys. Acta 1010, 131-139.
16. Nishizuka, Y. (1986) Science 233, 305-312.
17. Strahle, U., Schmid, W., and Schutz, G. (1988) The EMBO J. 7, 3389-3395.