EFFECT OF NICARDIPINE, A CALCIUM ANTAGONIST, ON INDUCTION OF PEROXISOMAL ENZYMES BY DEHYDROEPIANDROSTERONE SULFATE IN CULTURED RAT HEPATOCYTES

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ABSTRACT — We examined the effect of nicardipine, a calcium antagonist, on the induction of peroxisomal enzymes, such as acyl-CoA oxidase and carnitine acetyltransferase, by dehydroepiandrosterone sulfate (DHEAS) and clofibric acid (CPIB), in primary cultured rat hepatocytes. Peroxisomal β -oxidation and carnitine acetyltransferase activities were increased 11- and 20-fold, respectively, after 5 days of treatment with DHEAS (40 μ M). However, 60 μ M nicardipine significantly suppressed the induction of both of these activities by DHEAS to about 2-fold that of the control. This suppression was found to be both dose- and time-dependent. Immunoblot and Northern blot analyses of acyl-CoA oxidase revealed that suppression by nicardipine of the induction of peroxisomal β -oxidation activity would be responsible for an increase in the amount of mRNA. In addition, the manner in which nicardipine suppressed the induction of peroxisomal β -oxidation and carnitine acetyltransferase activity, was similar to that of clofibric acid. These findings suggest that in the calcium-dependent pathway, the mechanism for the induction of peroxisomal enzymes by DHEAS is basically the same as that by clofibric acid, a typical peroxisome proliferator. The present results also support our previous hypothesis that calcium may play an important role in the induction of these enzymes by peroxisome proliferators.

KEY WORDS : Peroxisome proliferator, Dehydroepiandrosterone, Nicardipine, Hepatocarcinogenesis, Peroxisomal enzyme

INTRODUCTION

Peroxisome proliferators include structurally unrelated compounds such as hypolipidemic drugs (Svoboda and Azarnoff, 1966 ; Reddy and Krishnakantha, 1975), industrial plasticizers

(Kluwe *et al.*, 1985) and herbicide solvents (Kawashima *et al.*, 1984). Despite being non-genotoxic compounds, peroxisome proliferators have a high incidence of hepatocarcinogenicity in rodents (Reddy and Lalwani, 1980). However, the mechanism of carcinogenesis is not fully understood.

Signal transduction, regulated by intracellular calcium concentration or protein phosphoryla-

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tion, is related to such important biological processes as cell proliferation and differentiation. Therefore, the changes in signal transduction could cause carcinogenesis. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), known to be a protein kinase C activator (Castagna *et al.*, 1982), promotes skin tumors in mice (Berenblum, 1975). Peroxisome proliferators induce pleiotropic responses that are related to the carcinogenic process such as increases in the peroxisome population, induction of peroxisomal β -oxidation enzymes and cell proliferation in the liver (Reddy and Lalwani, 1980; Lazarow and De Duve, 1976). Previous reports showed that peroxisome proliferators increased the calcium concentration in hepatocytes (Ochsner *et al.*, 1990; Bennett and Williams, 1992), and that the induction of peroxisomal β -oxidation by clofibrate was suppressed by some calcium antagonists, such as nicardipine and nifedipine (Watanabe and Suga, 1988; Itoga *et al.*, 1990). Therefore, calcium-related signal transduction may be important to the pleiotropic responses induced by peroxisome proliferators.

Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) are major adrenal secretory steroid in humans (Baulieu and Dray, 1963). DHEA is known to inhibit chemically induced tumors in the lung and colon of mice and to block phorbol ester promotion of skin tumors in mice (Schwarz and Tannen, 1981; Nyce *et al.*, 1984; Pashko *et al.*, 1984). Recently, it has been reported that DHEA induced peroxisome proliferation and peroxisomal β -oxidation enzymes (Leighton *et al.*, 1987; Yamada *et al.*, 1991) and, furthermore, that long-term treatment with DHEA induced a high incidence of hepatocarcinomas in male rats (Rao *et al.*, 1992; Hayashi *et al.*, 1994). However, DHEA does not activate the peroxisome proliferator-activated receptor (PPAR) which is known to be activated by typical peroxisome proliferators such as clofibric acid and Wy-14,643, regulating gene expression of acyl-CoA oxidase (Issemann and Green, 1990; Gottlicher *et al.*, 1992; Bocos *et al.*, 1995). Therefore, the mechanism of the induction of peroxisomal enzymes or hepatocarcinogenesis by DHEA may be different from that of typical peroxisome proliferators.

In the present study, in order to clarify whether calcium is related to the induction of

peroxisomal enzymes by DHEA, we examined the effect of nicardipine, a calcium antagonist, on the induction of peroxisomal enzymes by both DHEAS and clofibric acid (CPIB), a typical peroxisome proliferator. Since DHEAS was an actual active compound responsible for peroxisomal enzyme induction (Yamada *et al.*, 1992), DHEAS has been used instead of DHEA in this study.

MATERIALS AND METHODS

Primary cultured hepatocytes and treatment

Hepatocytes were isolated from male Wistar rats (200–250g) by a collagenase perfusion method (Seglen, 1976). The isolated hepatocytes in Leibovitz's L-15 medium (Flow Laboratories, Irvine, UK) containing 5% fetal bovine serum (FBS), 1 μ M dexamethasone, 1 μ M insulin, 100 U/ml penicillin G, 75 U/ml streptomycin sulfate, 100 U/ml kanamycin sulfate and 28 mM Hepes, were plated onto 35 mm plastic dishes coated with type I collagen. Cells were seeded at a density of 1.0×10^5 cells/cm² and allowed to attach to the dishes for 3 hr at 37°C in a humidified air atmosphere.

Dehydroepiandrosterone sulfate (DHEAS, 40 μ M), clofibric acid (CPIB, 250 μ M) or nicardipine (10–80 μ M), were dissolved in dimethylsulfoxide (0.4% (v/v), DMSO) and added to the culture medium. The medium and agents were changed daily. After exposure for an appropriate period, the cells from three dishes were harvested with 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. After disruption of cells by ultra sonication, the homogenates were used for assays of enzyme activity and immunoblot analysis.

Assay methods of enzyme activity

Peroxisomal β -oxidation activity was determined by the method of Lazarow and De Duve (1976), with palmitoyl-CoA as a substrate. The activity of carnitine acetyltransferase (CAT) was measured spectrophotometrically at 412 nm using the method described by Markwell *et al.* (1973).

Protein contents

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum

albumin as a standard.

Immunoblot analysis

The cell homogenates (10 μ g) were subjected to sodium dodecyl sulfate slab gel electrophoresis (Laemmli, 1970), transferred to nitrocellulose paper (Towbin *et al.*, 1979) and reacted with polyclonal anti-acyl-CoA oxidase (AOX) primary antibody (1 : 200 dilution) and anti-peroxisomal bifunctional enzyme (PBE, 1 : 200 dilution) for 1 hr at room temperature. The antigen-antibody complexes were visualized by the immunoperoxidase method using 3,3'-diaminobenzidine as a reagent.

RNA isolation and Northern blot analysis

Total RNA was isolated from the frozen tissue by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). Purification of poly(A)⁺RNA was carried out using Oligotex-dT30 (Takara Shuzo Co., Shiga, Japan). In a 1% agarose formaldehyde gel, 20 μ g total RNA was electrophoresed and blotted onto a Nylon membrane (Amersham, UK), which was then probed with 2.0 kbp rat AOX (a gift from Prof. T. Osumi, Himeji Institute of Technology, Hyogo, Japan) ³²P-labeled by a Megaprime DNA labeling system (Amersham, UK), washed, and exposed on X-ray film with an intensifying screen at -80°C .

Statistical analysis

Data were expressed as the mean $\pm$ SD with statistical significance determined by the Student's t-test.

RESULTS

As shown in Fig. 1, we examined whether nicardipine affected the induction of peroxisomal β -oxidation and CAT by DHEAS and CPIB in primary cultured rat hepatocytes. No changes in peroxisomal β -oxidation or CAT activities were observed in only nicardipine treated hepatocytes up until a concentration of 60 μM . At 80 μM , peroxisomal β -oxidation and CAT activity decreased 30 and 57%, respectively, as compared to treatments without nicardipine. In co-treatment with DHEAS and nicardipine, the induction of peroxisomal β -oxidation and CAT activity by

Fig. 1. Effect of nicardipine on the induction of peroxisomal β -oxidation and CAT by DHEAS and CPIB in cultured rat hepatocytes. (A) peroxisomal β -oxidation activity, (B) CAT. □; Control, ■; DHEAS, ▨; CPIB. The control levels of peroxisomal β -oxidation and CAT activities were 0.96 ± 0.036 and 2.87 ± 1.03 nmol/min/mg protein, respectively. The results were expressed as the mean $\pm$ SD of three dishes. Statistical significance; ^a $P < 0.05$ vs. DHEAS only, ^b $P < 0.05$ vs. CPIB only.

DHEAS was significantly decreased. The suppressive effect on both activities was first observed with the 10 μM nicardipine-treatment. Suppression was dependent on the concentration of nicardipine. Maximum suppression of the induction of peroxisomal β -oxidation and CAT, 14 and 16% respectively, was observed at a concentration of 60 μM . The induction of both

Fig. 2. Suppressive effect of nicardipine on peroxisomal β -oxidation and CAT by DHEAS and CPIB in cultured rat hepatocytes. (A) peroxisomal β -oxidation, (B) CAT. $\diamond$; nicardipine (40 μ M), $\blacksquare$; DHEAS, $\square$; DHEAS+nicardipine, $\bullet$; CPIB, $\circ$; CPIB+nicardipine. The control levels (day 0) of peroxisomal β -oxidation and CAT activities were 3.13 ± 0.21 and 7.84 ± 0.49 nmol/min/mg protein, respectively. The results were expressed as the mean $\pm$ SD of three dishes. Statistical significance; ^a $P < 0.05$ vs. DHEAS, ^b $P < 0.05$ vs. CPIB.

Fig. 3. Immunoblot analysis of acyl-CoA oxidase (AOX) and peroxisomal bifunctional enzyme (PBE), in cultured rat hepatocytes. The upper panel shows the immunoblot of AOX (72-, 52- and 21-kDa subunits) and the lower panel shows the immunoblot of PBE (78-kDa) after 5 days of treatment with DHEAS, CPIB or nicardipine (40 μ M). Lane 1; Control, lane 2; DHEAS, lane 3; DHEAS+nicardipine, lane 4; CPIB, lane 5; CPIB+nicardipine. Each lane contains 10 μ g protein.

activities was also suppressed by nicardipine, to the same degree, in the CPIB treated group.

Time-dependent changes in the activities of peroxisomal β -oxidation and CAT after co-

treatment with DHEAS or CPIB and nicardipine are shown in Figs. 2-A and -B. The control level of peroxisomal β -oxidation in the culture decreased from 3.13 ± 0.21 to 0.69 ± 0.17 nmol/min/mg protein, while the control level of CAT remained constant. At 5-day treatment with DHEAS, peroxisomal β -oxidation activity had increased 17-fold in cultured rat hepatocytes. This increase was shown to be time-dependent manner. Co-treatment with DHEAS and nicardipine significantly suppressed the induction of this activity. After 3 to 5-day co-treatment with DHEAS and nicardipine, the suppression of enzyme induction was 85 to 39%, as compared with DHEAS treated hepatocytes. CAT activity was increased 14-fold by DHEAS-treatment after 5 days. Suppression of the induction of CAT was also observed at 3-day treatment with DHEAS and nicardipine. In the CPIB-treated group, the suppression of peroxisomal β -oxidation and CAT activity by nicardipine showed the same pattern as that for DHEAS. Hepatocytes were treated with 40 μ M DHEAS and 40 μ M nicardipine for 5 days, and changes in the amounts of peroxisomal β -oxidation relative enzyme proteins were examined by immunoblot analysis (Fig. 3). Acyl-CoA oxidase (AOX) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE), which are constituents of the peroxisomal β -oxidation system, was in-

Fig. 4. Northern blot analysis of acyl-CoA oxidase mRNA in cultured rat hepatocytes. The upper panel shows the Northern blot of acyl-CoA oxidase mRNA after 5 days of treatment with DHEAS, CPIB or nicardipine (40 μ M). In the lower panel, the results of ethidium bromide staining of the 28 S and 18 S rRNA bands are shown. Lane 1; Control, lane 2; DHEAS, lane 3; CPIB, lane 4; DHEAS + nicardipine, lane 5; CPIB + nicardipine. Each lane contains 20 μ g total RNA that was applied on to the agarose gel.

creased in the DHEAS treated cells. On the contrary, nicardipine significantly suppressed the induction of the amounts of both enzyme proteins. Figure 4 shows the results of Northern blot analysis of the mRNA obtained from the hepatocytes treated with DHEAS and nicardipine. After 5 days of treatment with DHEAS, the amount of AOX mRNA was significantly increased, while the induction was suppressed by nicardipine. These changes were similar to those in the peroxisomal β -oxidation activity and the amount of AOX protein. In addition, the manner of this suppression was similar to that of clofibric acid.

DISCUSSION

Our previous studies have revealed that the induction of peroxisomal enzymes by clofibric acid was suppressed by nicardipine, a calcium antagonist (Watanabe and Suga, 1988; Itoga *et al.*, 1990), suggesting that intracellular calcium

plays an important role in the induction of peroxisomal enzymes by peroxisome proliferators. DHEA is a new peroxisome proliferator which has a high incidence of hepatocarcinogenicity and a chemical structure different from that of typical peroxisome proliferators (Rao *et al.*, 1992; Hayashi *et al.*, 1994). To clarify whether calcium is related to peroxisomal enzyme induction by DHEA, we examined the effect of nicardipine, a calcium antagonist, on the induction of peroxisomal enzymes by both DHEAS and clofibric acid, a typical peroxisome proliferator.

Nicardipine significantly suppressed the induction of peroxisomal β -oxidation and CAT by DHEAS in both a dose- and time-dependent manner. The suppression of induced peroxisomal β -oxidation activity and enzyme proteins was responsible for a reduction of steady state acyl-CoA oxidase mRNA. Nicardipine is well known to be a calcium antagonist and to decrease cellular calcium concentration. Since calcium is closely related to signal transduction system, changes in intracellular calcium concentration should affect various biological processes such as transcription, cell proliferation and differentiation. Our present findings suggest that calcium is an important factor in regulating the transcription of enzyme-induction by DHEAS. In fact, peroxisome proliferators increase intracellular calcium concentration (Ochsner *et al.*, 1990; Bennett and Williams, 1992) and activate calcium dependent protein kinase such as protein kinase C (Bojes *et al.*, 1992).

In 1990, Issemann and Green reported on a nuclear hormone receptor which could be activated by several different peroxisome proliferators, being called peroxisome proliferator activated receptor (PPAR). Although both DHEAS and DHEA induce peroxisomal enzymes, neither of these compounds has been able to activate PPAR (Gottlicher *et al.*, 1992; Bocos *et al.*, 1995). These findings lead us to the speculation that the induction of peroxisomal enzymes by DHEA or DHEAS is caused by another PPAR or a receptor system other than that of PPAR. In fact, DHEAS specific binding protein which does not bind to clofibric acid and ciprofibrate is found (Yamada *et al.*, 1994). In the present study, we showed that nicardipine

suppressed the induction of peroxisomal enzymes by DHEAS and that the manner of this suppression was similar to that of clofibric acid which can activate PPAR. Previous reports showed that peroxisome proliferators increased the calcium concentration in hepatocytes (Ochsner *et al.*, 1990; Bennett and Williams, 1992), and stimulated protein kinase C which required calcium to activate (Bojes *et al.*, 1992). We also found that protein kinase C inhibitors suppressed the induction of peroxisomal β -oxidation by both DHEAS and clofibric acid (unpublished data). Therefore, in calcium-dependent signal transduction pathways, the mechanism for the induction of peroxisomal enzymes by DHEAS may be the same as that occurred by clofibric acid or other typical peroxisome proliferators.

In conclusion, the induction of peroxisomal enzymes by DHEAS was suppressed by a calcium antagonist as well as by clofibric acid. The mechanism for the induction of peroxisomal enzymes by DHEAS was basically the same as that by clofibric acid, a typical peroxisome proliferator. Furthermore, intracellular calcium may play an important role in the induction of these enzymes by peroxisome proliferators.

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