Calmodulin antagonists increase the amount of mRNA for the low-density-lipoprotein receptor in skin fibroblasts

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The effects of calmodulin antagonists on the amount of LDL receptor (LDL-R) mRNA in cultured human fibroblasts was examined by hybridization with a fragment of LDL-R cDNA. In a 'Northern' blot the fragment hybridized to a 5.3-kilobase RNA, as expected for LDL-R mRNA. The concentration of this RNA was increased in preparations from cells that were treated with trifluoperazine or W-7 [*N*-(6-aminoethyl)-5-chloronaphthalene-1-sulphonamide]. The selectivity of the increase was established by using a probe for β -actin mRNA. In dot-blot hybridization it was observed that the calmodulin antagonists cause 2–4-fold relative increase in the amount of LDL-R mRNA.

INTRODUCTION

The low-density-lipoprotein (LDL) receptor, a trans-membrane cell-surface glycoprotein, mediates endocytosis of macromolecular lipoprotein ligands. The regulation of the activity of the LDL receptor has been studied extensively in both cultured cells and experimental animals. Although the primary structure (Yamamoto *et al.*, 1984) and synthesis of the LDL receptor (Goldstein *et al.*, 1985) and the organization of the gene encoding this glycoprotein (Südhof *et al.*, 1987) have been elucidated, little is known about the mechanisms of the regulation of the synthesis of the LDL receptor. In human skin fibroblasts, depletion of cholesterol enhances, and loading with cholesterol lowers, the activity of the LDL receptor (Brown *et al.*, 1981). The activity of the LDL receptor (Kovanen *et al.*, 1981; Chao *et al.*, 1979) and the amount of its mRNA (Ma *et al.*, 1986*a,b*) are enhanced in livers of rats, hamsters and rabbits treated with mevinolin (Ma *et al.*, 1986*b*) or with pharmacological doses of 17-ethinyloestradiol (Ma *et al.*, 1986*a*). Drugs perturbing the intracellular Ca^{2+} functions also modulate the expression of the LDL receptor. Both the Ca^{2+} -entry blockers verapamil and diltiazem (Filipovic & Buddecke, 1986*a*) and the calmodulin antagonists trifluoperazine and W-7 (Filipovic & Buddecke, 1986*b*) increase the apparent rate of synthesis and the endocytic capacity of the LDL receptors in human skin fibroblasts.

In the present study it is shown that in human fibroblasts treated with calmodulin antagonists the induced stimulation of LDL-receptor synthesis is correlated with a selective increase in the amount of LDL-receptor-specific (LDL-R) mRNA.

MATERIALS AND METHODS

Cell culture

Human skin fibroblasts were cultivated from skin explants of a healthy donor and grown as described by Cheung (1980). Culture flasks (75 cm²) were seeded with

10⁵ cells in Eagle's minimum essential medium (LS Labor Service, München, Germany) containing 10% (v/v) fetal-calf serum (Boehringer-Mannheim, Mannheim, Germany). Confluent culture obtained after 5–6 days were washed once with phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate, pH 7.4) and incubated in a medium containing 5% (v/v) lipoprotein-deficient human serum for 24 h. After addition of calmodulin antagonists or other effectors, the incubation was continued for further 6 h.

Isolation and hybridization of RNA

Total RNA was isolated from cultured human fibroblasts by repeated precipitation with ethanol from 8 M-guanidinium chloride (Adams, 1977). For Northern-blot analysis, equal amounts of RNA, determined at 260 nm, were denatured with formaldehyde and electrophoresed in 1%-agarose gels in the presence of formaldehyde (Maniatis *et al.*, 1982). The RNA was transferred to nylon membranes (Hybond, from Amersham-Buchler, Braunschweig, Germany) by capillary blotting with 20 × SSC (Maniatis *et al.*, 1982) for 12 h, and fixed by u.v. irradiation. Dot-blot analysis was performed on nylon membranes with serial dilutions of glyoxal-denatured RNA (Maniatis *et al.*, 1982). The membranes were air-dried, irradiated with u.v. light and heated at 80 °C for 1 h. The pre-hybridization and hybridization incubations were performed essentially as described by Thomas (1980). The pre-hybridization buffer contained 50% (v/v) formamide (deionized), 50 mM-sodium phosphate, 0.1% SDS, 5 × SSC, 0.1% (w/v) each of bovine serum albumin, Ficoll and polyvinylpyrrolidone, and 0.5 mg of sonicated denatured salmon sperm DNA/ml. The hybridization was performed in the same buffer with 2 ng of labelled DNA probes/ml (see below) at 42 °C for 12 h. The membranes were washed with 2 × SSC containing 0.1% SDS at room temperature for 1 h and then at 60 °C for 20 min. The radioactivity was detected with Kodak XR5 films and Cronex intensifying screens (DuPont, Bad Nauheim, Germany) at –70 °C.

Abbreviations used: LDL, low-density lipoprotein; LDL-R mRNA, LDL-receptor mRNA; kb, kilobases; SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0.

Fig. 1. Northern-blot analysis of LDL-R and β -actin mRNAs

Total RNA isolated from human skin fibroblasts (20 μ g/lane) was separated in formaldehyde/1% agarose gel, blotted on to nylon membranes and hybridized sequentially with LDL-R (a) and β -actin (b) probes. The positions of 28 S and 18 S rRNA were determined by staining a parallel lane with ethidium bromide. Pretreatment of cultured cells was as follows: C, control; T, 25 μ M-trifluoperazine; H, 1 μ g of 25-hydroxycholesterol/ml; W, 25 μ M-W-7.

For hybridization, a fragment of the LDL-receptor cDNA and, as a control, a β -actin-pseudogene DNA were used. The LDL-receptor probe was prepared by digesting the plasmid pLDL3 (American Type Culture Collection, Rockville, MD, U.S.A.) with the restriction endonucleases *Eco*RI and *Xho*I, separation of the fragments by agarose-gel electrophoresis and recovering the fragment with DEAE-cellulose paper (Dretzen *et al.*, 1981). Similarly, human β -actin-pseudogene DNA was isolated as a *Hind*III fragment from the plasmid pHAc-69A (Moss & Gallwitz, 1983). The fragments were radioactively labelled by random primed synthesis (Feinberg & Vogelstein, 1983) with [α - 32 P]dATP (110 TBq/mmol; Amersham-Buchler) to a specific radioactivity of $(2-3) \times 10^9$ d.p.m./ μ g.

RESULTS

Human skin fibroblasts were grown to confluence in lipoprotein-free medium. The cells were incubated in the absence or presence of trifluoperazine and W-7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide] for 24 h, and RNA was isolated. 25-Hydroxycholesterol, which is known to suppress the synthesis of LDL receptor (Goldstein *et al.*, 1985), was used as negative control.

The RNA was subjected to Northern-blot analysis. It was probed sequentially with 32 P-labelled LDL-R cDNA and β -actin-pseudogene DNA fragments. As shown in Fig. 1, the LDL-R cDNA hybridized to a RNA that migrated behind the 28 S rRNA standard. By the criterion of size, the signal corresponded to human LDL-R mRNA (Yamamoto *et al.*, 1984). The intensities of the hybridization signals indicated that RNA isolated from

cells pretreated with trifluoperazine or W-7 contained more LDL-R mRNA than did RNA isolated from control cells. In Fig. 1 the amount of LDL-R mRNA seems to be increased by at least an order of magnitude after the treatment with 25 μ M-trifluoperazine. In a similar experiment this relative change as compared with the amount of β -actin mRNA was only 3–5-fold. In cells that had been pretreated with 25-hydroxycholesterol, the amount of LDL-R mRNA was decreased as compared with control.

If the same blot was hybridized to the β -actin probe, two signals were detected (Fig. 1b). The major signal corresponded to a RNA species of 2.0 kb and the minor one to approx. 3.8 kb. The major species had the size expected for β -actin mRNA (Gunning *et al.*, 1983). From the comparison of the hybridization signals obtained with the two probes, it appeared that the increases in LDL-R mRNA brought about by trifluoperazine and W-7 were selective.

Semi-quantitative estimations of LDL-R mRNA are presented below, which were obtained by dot-blot hybridization. Fig. 2 shows the result of hybridization of serially diluted RNA from control and calmodulin-antagonist-pretreated cells with probes for LDL-R and β -actin mRNAs. From the comparison of the signal intensities obtained at different dilutions with the two probes, it was concluded that the pretreatment of fibroblasts with trifluoperazine enhanced the relative concentration of LDL-R mRNA 2–4-fold and the pretreatment with W-7 did so about 2-fold. In Fig. 3 a dependence of the increase in LDL-R mRNA on the concentration of trifluoperazine is demonstrated. The increase was maximum in cells that were treated with 12 μ M-trifluoperazine. The attenuation of the enhance-

Fig. 2. Dot hybridization of LDL-R and β -actin mRNAs from control and effector-pretreated fibroblasts

Total RNA extracted from the cells was dotted in serial dilutions on to nylon membranes and hybridized to denatured double-stranded ^{32}P -labelled probes as indicated. Pretreatment of cultured cells was as follows: C, control; T, 25 μM -trifluoperazine; W, 25 μM -W-7.

Fig. 3. Dose-dependent alterations of LDL-R mRNA amounts in skin fibroblasts pretreated with trifluoperazine

Serial dilutions of total cellular RNA were sequentially dot-hybridized with the β -actin and LDL-R probes.

ment at 25 μM -trifluoperazine resulted probably from toxic side effects of the drug, which had been reported by others (Hidaka *et al.*, 1981).

DISCUSSION

Our previous studies suggested that Ca^{2+} -calmodulin participates in down-regulation of LDL-receptor syn-

thesis in human skin fibroblasts (Filipovic & Buddecke, 1986b). This conclusion was based on the observation of a marked enhancement of the incorporation of [^{35}S]methionine into the LDL-receptor protein in cells treated with calmodulin antagonists W-7 and trifluoperazine. The half-life of the receptors was not changed by the treatments. The present data show that the calmodulin-antagonist-induced increase in LDL-receptor activity is correlated with a corresponding increase in LDL-R mRNA.

In additional experiments we observed that 25 μM -trifluoperazine increased LDL-R mRNA to a rather variable degree. We presume that at this concentration trifluoperazine has a toxic effect. In a dot-hybridization experiment (Fig. 3) examining the optimum concentration of trifluoperazine for the enhancement of LDL-R mRNA, 12 μM appeared to be the most effective. In dot-hybridization experiments the effect of trifluoperazine seemed to be less dramatic than in Northern blots, probably because of a higher background signal in the former.

It remains to be examined whether calmodulin antagonists increase the rate of the transcription or decrease the rate of degradation of LDL-R mRNA, or affect both. We cannot exclude the possibility that this effect is due to interactions of the antagonists with targets other than calmodulin, e.g. the glucocorticoid receptor (Van Bohemen & Rousseau, 1982). Nevertheless, because of the prominent role of LDL in the pathogenesis of vascular disease, it should be of interest to examine a possible link between activation of calmodulin in organs which utilize LDL and the deterioration of vessels.

Calmodulin has been recognized to play a central role in the processing of the Ca^{2+} signal (Means *et al.*, 1982) either by reversible association with target proteins or by stimulation of protein kinases (Klee & Newton, 1983). Calmodulin is present in the nucleus and modulates phosphorylation of several nuclear proteins that are possibly involved in the regulation of transcription (Bachs & Carafoli, 1987). In GH₃ cells the amount of prolactin mRNA is enhanced by epidermal growth factor, and this enhancement is abolished in the presence of calmodulin antagonists (White, 1985). Our results indicate that calmodulin antagonists selectively enhance the amount of the LDL receptor in cultured fibroblasts.

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