Involvement of Second Messengers in Regulation of the Low-Density Lipoprotein Receptor Gene

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Transcription of the low-density lipoprotein receptor (LDL-R) gene in the human monocytic leukemic cell line THP-1 and in the human hepatocarcinoma cell line Hep-G2 is regulated by second messengers of the diacylglycerol-protein kinase C (DAG-PKC), inositol 1,4,5-triphosphate-Ca²⁺, and cyclic AMP pathways. Exogenous phospholipase C (which releases DAG and inositol 1,4,5-triphosphate), PKC activators (phorbol esters and DAG), Ca²⁺ ionophores, and a cyclic AMP analog all transiently induced accumulation of LDL-R mRNA. The effects of these three signal-transducing pathways were to a large extent additive. Furthermore, PKC stimulation effected an increase in LDL binding, which suggested that the increase in LDL-R mRNA resulted in an increase in functional cell surface receptor activity. These results suggest that uptake of cholesterol by these cells is under control of both intracellular cholesterol levels and external signals.

Mammalian cells can obtain cholesterol necessary for synthesis of membranes, steroid hormones, and bile acids by de novo synthesis or uptake of lipoprotein-derived cholesterol via the low-density lipoprotein receptor (LDL-R) (12). Cells usually maintain a low rate of cholesterol synthesis and rely predominantly on the LDL-R pathway for their cholesterol needs. Synthesis of the LDL-R has been shown to be subject to finely tuned feedback inhibition by intracellular cholesterol (12). Recently, we demonstrated that LDL-R gene expression also is under the control of protein kinase C (PKC) and a labile negative regulatory protein (1).

Transduction of a wide variety of extracellular signals occurs either by membrane phosphoinositides and their breakdown products inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (3, 22) or via the adenylate cyclase-cyclic AMP (cAMP) system (16). Whereas DAG leads to activation of PKC, IP₃ triggers the release of Ca²⁺ from the endoplasmic reticulum, resulting in activation of Ca²⁺-calmodulin-dependent protein kinases. Activation of adenylate cyclase raises the level of cAMP, which activates cAMP-dependent kinases. These second messengers promote nuclear and cytoplasmic events required for cell proliferation and differentiation (14, 24). To gain further insight into regulation of LDL-R gene expression by second messengers, we investigated the effect of several compounds which selectively activate PKC, release intracellular Ca²⁺, or increase intracellular cAMP concentration on LDL-R mRNA levels in THP-1 and Hep-G2 cells. The results show that LDL-R gene expression is strongly stimulated by each of these second-messenger systems.

MATERIALS AND METHODS

Materials. Phorbol 12-myristate 13-acetate (PMA) was obtained from LC Services Corp. (Woburn, Mass.). A-23187 and dibutylrlyl cyclic 3',5'-monophosphate (dBcAMP) were from Sigma Chemical Co. (St. Louis, Mo.). Hybond-N nylon membranes for blotting of RNA were from Amersham Corp.,

Arlington Heights, Ill.), and the random-prime labeling kit, cycloheximide (CHX), and actinomycin D were from Boehringer Mannheim Biochemicals Corp. (Indianapolis, Ind.).

Cell culture. The human cell lines THP-1 (30) and Hep-G2 were obtained from the American Type Culture Collection (Rockville, Md.) and grown, respectively, in RPMI 1640 and in Eagle minimal essential medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂-95% air as described elsewhere (1).

Determination of LDL binding. LDL (*d*, 1.019 to 1.063 g/ml) was prepared and radioiodinated as described previously (13). LDL binding was measured by the method of Goldstein et al. (11) as described previously (6). After incubation of cells at 4°C for 4 h with 5 µg of ¹²⁵I-labeled LDL per ml, the cells were washed extensively and dissolved in 0.1 M NaOH for determination of LDL binding and protein content (19).

RNA analysis. Total RNA was isolated by the guanidine isothiocyanate-CsCl method (7) and analyzed either by electrophoresis through 1.2% agarose-formaldehyde gels (17) followed by capillary transfer to nylon membranes or by dot blot hybridization as described previously (1a). LDL-R mRNA was detected by using the ³²P-labeled (random priming; specific activity, approximately 10⁹ cpm/µg) 1.9-kilobase *Bam*HI fragment (which contains both 3' coding and noncoding sequences) of the human LDL-R cDNA clone pLDLR-3 (kindly provided by J. L. Goldstein and M. S. Brown and described by Yamamoto et al. [33]). A 0.75-kilobase *Eco*RI-*Hind*III fragment of pA1, a chicken beta-actin cDNA clone (9), was used as the control probe. Quantitation of specific hybrids was made by densitometric tracing of autoradiographs in the linear range of the X-ray film.

Transcription rate assay. Isolation of nuclei and transcription run-on assays were performed as described by Nevins (21) except that nuclear RNA was extracted by the acid-guanidine thiocyanate-phenol-chloroform method (8). Filter prehybridization and hybridization were performed as for the RNA blots (1). Equivalent amounts of labeled nuclear RNA (3 × 10⁶ cpm) were hybridized for 36 h at 42°C to three different amounts (1, 0.5, and 0.25 µg) of purified cloned cDNAs (described above) immobilized on Hybond nylon

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FIG. 1. Time course (A) and dose response (B) of LDL-R (●) and actin (○) mRNA accumulation after treatment of THP-1 cells (60×10^6) with A-23187, as determined by dot blot hybridization (insets). (C) RNA gel blot analysis of RNA (20 μg) from untreated cells (lane 1) or THP-1 cells exposed for 2 h with 5 μg of A-23187 per ml (lane 2). kb, Kilobases.

filters. The filters were washed and treated with RNase before autoradiography.

RESULTS

Transient induction of LDL-R gene transcription in THP-1 and Hep-G2 cells by mobilization of Ca^{2+} . The Ca^{2+} pathway of signal transduction is physiologically triggered by the release of IP_3 , which mobilizes membrane-bound Ca^{2+} . Involvement of this pathway in the regulation of the LDL-R gene was investigated by using the Ca^{2+} ionophore A-23187 to mimic the effect of IP_3 in THP-1 and Hep-G2 cells. A transient and marked (17-fold) increase in the steady-state level of LDL-R mRNA in THP-1 cells, a human monocytic cell line, was observed within 3 h of addition of A-23187, whereas actin mRNA levels did not change (Fig. 1A). Induction of LDL-R mRNA was dependent on the concentration of A-23187 (Fig. 1B), which, unlike PMA, did not induce THP-1 to differentiate into macrophagelike cells. Northern (RNA) blot analysis of RNA from THP-1 cells before and after A-23187 addition (Fig. 1C) showed an increase in the level of an mRNA species corresponding in size (5.3 kilobases) to LDL-R mRNA in Hep-G2 cells and to that previously isolated from bovine adrenal cells (26). Superinduction of the LDL-R mRNA level (data not shown) was observed when A-23187 was added together with CHX, which also has been shown (1) to be an inducing agent.

Increased steady-state levels of mRNA could be the result of either increased transcription or enhanced stability of the mRNA. Treatment of THP-1 cells with actinomycin D (5 $\mu\text{g/ml}$) for 90 min before addition of A-23187 blocked induction of LDL-R mRNA, whereas only a minimal effect was seen on actin mRNA; this result suggested that LDL-R mRNA induction was at the level of transcription (data not shown). Furthermore, transcription rates assayed directly, by nuclear run-on experiments, showed an approximately

2.5-fold increase in the transcription rate of the LDL-R gene after treatment with A-23187 (Fig. 2).

Since the liver plays a key role in cholesterol excretion and lipoprotein catabolism, we also tested whether LDL-R mRNA steady-state levels were influenced by A-23187 and PMA in the human hepatocarcinoma cell line Hep-G2. Addition of A-23187 or PMA to Hep-G2 cells resulted in an approximately three- or fivefold increase in LDL-R mRNA steady-state levels. The effect of A-23187 on LDL-R mRNA in Hep-G2 cells was slower than that in THP-1 cells, since LDL-R mRNA levels only started increasing 2 h after addition of A-23187 (Fig. 3). We next investigated whether increased levels of LDL-R mRNA resulted in increased LDL binding to the cell surface. Since THP-1 cells grow in suspension, it is difficult to perform LDL-R binding studies. In Hep-G2 cells, PMA treatment resulted in a transient threefold increase in LDL binding (from 10.3 to 30 ng/mg of cell protein at 12 h and 18 ng/mg of cell protein 24 h after addition of PMA). This finding suggests that changes in LDL-R mRNA levels, such as those seen in this study, might have important physiological consequences.

Induction of LDL-R by Ca^{2+} does not involve stimulation of PKC or the Ca^{2+} -calmodulin-dependent kinase. Whereas low concentrations of Ca^{2+} ionophore stimulate only the calmodulin-dependent protein kinase, high concentrations stimulate PKC as well (3, 22). To evaluate the independent roles of calmodulin-dependent protein kinase and PKC, we first tested whether Ca^{2+} mobilization and PKC activation had additive effects on LDL-R mRNA levels when both agents were added to Hep-G2 and THP-1 cells concurrently for 2 h at the maximum effective concentrations. PMA and A-23187 independently led to an increase in LDL-R mRNA levels both in Hep-G2 cells (threefold for A-23187 and sevenfold for PMA) and THP-1 cells (fivefold for A-23187 and fourfold for PMA) (Fig. 4). A-23187 (5 $\mu\text{g/ml}$) added together with

FIG. 2. Analysis of transcription rate of the LDL-R in THP-1 cells. Labeled RNA was obtained from nuclei prepared from THP-1 cells before treatment ($\square$) or 1 h after treatment with PMA (1.6×10^{-7} M), CHX (10 μ g/ml), A-23187 (5 μ g/ml), or dBcAMP (100 μ M) ($\boxplus$). The RNAs were hybridized with different concentrations of isolated cDNA inserts of LDL-R, beta-actin (1 and 0.5 μ g of DNA), or pGEM vector (5 and 2.5 μ g of DNA) as described in Materials and Methods. Values were normalized to hybridization to the actin probe, which did not change appreciably.

PMA (1.6×10^{-7} M) resulted in superinduction in Hep-G2 but not THP-1 cells (14-fold increase in steady-state mRNA levels; Fig. 4). However, treatment of THP-1 cells with slightly lower concentrations of A-23187 (3 μ g/ml) and PMA (8×10^{-8} M), which still elicited maximal induction of LDL-R mRNA, resulted in a marked additive effect (Fig. 4).

FIG. 3. Induction of LDL-R mRNA by A-23187 in Hep-G2 cells. Confluent Hep-G2 cells (15×10^6) were exposed for the indicated times to A-23187 (5 μ g/ml; $\circ$), PMA (1.6×10^{-7} M; $\bullet$), or medium without additions ($\triangle$) for evaluation of LDL-R mRNA as described in Materials and Methods. Values were normalized to actin mRNA levels.

FIG. 4. Additive effects of A-23187 and PMA on LDL-R mRNA accumulation in Hep-G2 and THP-1 cells. Symbols: $\square$, Hep-G2 cells treated with A-23187 (5 μ g/ml) and PMA (1.6×10^{-7} M); $\boxplus$, THP-1 cells treated with A-23187 (3 μ g/ml) and PMA (8×10^{-8} M). RNA was isolated and analyzed as described in Materials and Methods.

Furthermore, after prolonged treatment of cells with PMA (which results in depletion of cellular PKC [23]), LDL-R mRNA could still be induced (fourfold) by the calcium ionophore (Fig. 5), whereas restimulation of these cells with PMA had no effect on LDL-R mRNA levels. These data suggest that Ca^{2+} mobilization and stimulation of PKC have at least partially independent effects on LDL-R gene expression.

Induction of LDL-R mRNA by cAMP. In a variety of cell types, cyclic nucleotides are known to serve as second messengers in the transduction of hormonal signals (16, 24). It was therefore of interest to investigate whether cAMP might be involved in regulation of LDL-R mRNA expression. Exposure of THP-1 cells to dBcAMP resulted in a fourfold transient increase in LDL-R mRNA transcription (Fig. 2) and a twofold increase in the steady-state level (data not shown). A twofold superinduction of LDL-R mRNA levels was observed when THP-1 cells were treated with both dBcAMP and CHX (which also has been shown [1] to induce LDL-R mRNA expression) (data not shown).

DISCUSSION

Hormones and growth factors regulate cell proliferation and function by binding to cell surface receptors and trigger the formation or release of second-messenger molecules from the membranes. We have shown elsewhere that activation of PKC (by DAG and PMA) transiently induces the LDL-R gene in THP-1 cells (1). Evidence is now presented to show that such induction also could be achieved by treatment with either the calcium ionophore A-23187 or dBcAMP and that the induction is at the level of transcription. It is therefore concluded that LDL-R gene expression is under control of extracellular signals that are transduced by the second-messenger systems of DAG-PKC, IP_3 - Ca^{2+} and cAMP. Induction of LDL-R mRNA in Hep-G2 cells by the

FIG. 5. Demonstration that induction of LDL-R mRNA by A-23187 is independent of the activation of PKC. THP-1 cells were exposed for 72 h to PMA (1.6×10^{-7} M) to deplete them of PKC. Cells then were rinsed twice with phosphate-buffered saline and refed with medium containing either PMA (1.6×10^{-7} M; ●) or A-23187 (5 μ g/ml; ○). LDL-R mRNA levels were normalized to actin mRNA levels. The inset shows the autoradiograph.

DAG-PKC second-messenger system also resulted in an increase in LDL-R activity.

The induction of LDL-R mRNA by A-23187 was at least partially independent of PKC-mediated activation of the LDL-R gene. In contrast to the very transient induction observed after treatment of THP-1 cells by PMA, the effect of A-23187 was more sustained, resulting in accumulation of LDL-R mRNA for a longer period of time (1 to 2 h for PMA versus 3 to 4 h for A-23187). In addition, the effects of A-23187 and PMA on LDL-R mRNA were additive (Fig. 4), and A-23187 was still able to induce LDL-R mRNA levels in PKC-depleted THP-1 cells. Therefore, it is concluded that both PKC and Ca^{2+} mobilization, the two pathways activated after inositol phospholipid turnover, induce LDL-R mRNA in THP-1 cells independently. Partially independent effects of PMA and A-23187 have previously been described for transcriptional activation of several genes (25), including *c-myc* (18) and *c-fos* (4, 28). It has also been shown that different 5' *cis*-acting sequences of the *c-fos* gene are involved in mediating the effects of PMA and A-23187 in HeLa, A-431 (10), and PC12 (27) cells. The mechanism by which Ca^{2+} mobilization leads to an increase in LDL-R gene transcription is unclear at present. Calmodulin and calmodulin- Ca^{2+} -dependent kinases have been shown to play important roles in mediating the effects of the Ca^{2+} signal (20), since they modulate phosphorylation of several nuclear proteins that may be involved in transcriptional regulation (2).

The adenylate cyclase pathway also regulates LDL-R mRNA levels in THP-1 cells, as evidenced by the inductive effects of dBcAMP. It was previously reported that dBcAMP lowered LDL-R activity in cultured human fibroblasts and smooth muscle cells (29). Since LDL-R activity in that study was measured at 24 h after addition of dBcAMP, those data do not conflict with the transient increase in LDL-R mRNA seen in THP-1 cells.

Since the cAMP, DAG-PKC, and IP_3 - Ca^{2+} pathways all

lead to protein phosphorylation, it is likely that the distinct phosphorylation events initiated by different messengers may overlap at one or several points, resulting in cross-talk between the different systems (5, 31, 34). This cross-talking is suggested by the following observations: the PMA-induced phosphorylation of the catalytic subunit of adenylate cyclase (34), the utilization of the AP-2 *cis*-acting element in mediating both cAMP and phorbol ester gene induction (15; see reference 24 for a review), the cAMP-mediated translocation of PKC to the nucleus (5), and the PKC-induced phosphorylation of the cAMP-responsive-element binding protein (32). In this context, it is suggested that the partially additive stimulatory effects of the three pathways of signal transduction on the LDL-R gene could be the result of activation of partially overlapping subsets of transcription factors. It is also conceivable that the effect of these second messengers on LDL-R gene induction is indirect and occurs as a result of physiological changes that may deplete pools of intracellular cholesterol or mevalonate-derived products.

We observed previously that transcription of LDL-R was induced by CHX, which points to the involvement of a labile negative regulatory protein in the regulation of LDL-R mRNA levels (1). In this study, superinduction of LDL-R mRNA was observed when CHX was added together with either phospholipase C, A-23187, PMA, or dBcAMP, which suggests that this labile repressor protein is to a large extent independent of the action of these second messengers.

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