Stimulation of low-density lipoprotein uptake in HepG2 cells by epidermal growth factor via a tyrosine kinase-dependent, but protein kinase C-independent, mechanism

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Epidermal growth factor (EGF), a potent mitogenic polypeptide, stimulated the uptake and degradation of [³H]sucrose-labelled low-density lipoprotein (LDL) by HepG2 cells. The increase in LDL uptake was prevented by the presence of the tyrosine kinase inhibitor genistein. Activation of protein kinase C with phorbol 12-myristate 13-acetate (PMA) also stimulated the uptake of [³H]LDL by HepG2 cells. When EGF and PMA were added together, PMA increased the response to EGF in an additive

manner. The protein kinase C inhibitor Ro-31-8220 prevented the increase in LDL uptake caused by PMA, but did not affect EGF stimulation of LDL uptake. Similarly, down-regulation of protein kinase C activity by chronic treatment with PMA also did not affect the EGF stimulation of LDL uptake. These results suggest that the EGF stimulation of LDL uptake and degradation by HepG2 cells is mediated by a tyrosine kinase-dependent, but protein kinase C-independent, mechanism.

INTRODUCTION

The hepatic low-density lipoprotein (LDL) receptor is responsible for clearing around 70 % of plasma LDL from the circulation, and regulation of the level of LDL receptor expression is therefore a major factor in determining plasma LDL cholesterol concentration (Goldstein and Brown, 1977; Havel, 1986; Dietschy et al., 1993). The sterol-mediated down-regulation of cholesterol biosynthesis and of LDL receptor expression, following repression of transcription of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, HMG-CoA synthase and LDL receptor genes, is well established (Goldstein and Brown, 1977). However, cultured hepatocytes express LDL receptor activity in the presence of lipoproteins, suggesting that the suppression of LDL receptor activity in hepatocytes is less responsive to the external concentrations of lipoprotein cholesterol than in cultured fibroblasts (Havekes et al., 1983; Edge et al., 1986; Salter et al., 1987).

It has become increasingly apparent that LDL receptor expression is under the control of various growth factors and hormones. Receptor activity in a number of different cell types is increased by incubation with insulin (Chait et al., 1979; Salter et al., 1988; Wade et al., 1988), thyroid hormone (Chait et al., 1979; Salter et al., 1988; 1991) and by glucagon and adrenaline (Brown et al., 1989; Middleton and Middleton, 1992), whereas the synthetic glucocorticoid dexamethasone decreases receptor expression (Salter et al., 1988). Serum factors have also been shown to increase LDL receptor levels in HepG2 cells in a sterol-independent manner (Ellsworth et al., 1988, 1991). Growth factors, such as platelet-derived growth factor (PDGF), fibroblast-derived growth factor (FGF) and endothelial-cell-derived growth factor (ECCM), have been shown to increase LDL receptor expression in vascular smooth-muscle cells and cultured human fibroblasts (Chait et al., 1980, 1988; Witte et al., 1982; Davies and Kerr, 1982; Mazzone et al., 1989). Similarly, macrophage-derived factors such as oncostatin M, interleukin-6

and interleukin-1 α have been shown to increase LDL receptor expression in HepG2 cells (Grove et al., 1991). Oncostatin M is thought to increase LDL receptor expression via a tyrosine kinase and protein kinase C-dependent mechanism (Grove et al., 1992). The increase in LDL receptor expression due to growth factors is thought to be mediated at the level of LDL receptor mRNA transcription (Mazzone et al., 1989).

The binding of epidermal growth factor (EGF) to its receptor tyrosine kinase results in receptor autophosphorylation and activation of phospholipase C γ -1, which in turn leads to increased phosphoinositide turnover, activation of protein kinase C and release of Ca²⁺ from intracellular stores (Carpenter and Cohen, 1990). It is not known which of these signalling pathways is involved in the increased expression of the LDL receptor in response to EGF. It is clear that a number of second messengers, such as cyclic AMP, Ca²⁺ and phorbol esters (which can activate protein kinase C) can increase the expression of LDL receptor mRNA in THP-1 monocytic cells, and in HepG2 cells (Auwerx et al., 1989a,b). In this study we have examined the role of tyrosine kinases, and of protein kinase C, in the cell-signalling pathway induced by EGF which results in the stimulation of LDL receptor expression in HepG2 cells.

METHODS

Materials

Chemicals and radiochemicals were purchased from Sigma Chemical Co. and Amersham International respectively. Tissue-culture medium Williams E, PBS (Ca²⁺- and Mg²⁺-free), glutamine, penicillin/streptomycin, NaHCO₃, Hepes buffer, BSA (Cohn fraction V) and foetal-calf serum (FCS) were purchased from Gibco BRL, Paisley, Scotland, U.K. Tissue-culture plates (6-well) were purchased from Falcon. Mevinolin (lovastatin) was kindly given by Merck Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.

Abbreviations used: DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; FCS, foetal-calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoprotein; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; TCA, trichloroacetic acid.

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Preparation and labelling of LDL

LDL was prepared by the method of Chung et al. (1980), by using the rapid single-discontinuous-density-gradient ultracentrifugation process. The LDL was dialysed against 20 litres of PBS containing 1 mM EDTA. LDL was radiolabelled with [6,6'(n)-³H]sucrose, essentially as described by Pittman and Taylor (1986), except that [³H]sucrose (6.2 Ci/mmol) was used instead of [U-¹⁴C]sucrose. The specific radioactivity achieved by this process was around 20 d.p.m./ng of LDL protein. Over 95% of the radioactivity could be precipitated with 10% (w/v) trichloroacetic acid (TCA), and less than 5% could be extracted with chloroform/methanol (1:2, v/v).

Preparation and culture of HepG2 cells

HepG2 cells were cultured in 6-well tissue-culture plates until around 80% confluency was achieved. The cells were cultured in Williams E medium containing 10% FCS, glutamine (4 mM), NaHCO₃ (0.09%, v/v), Hepes (20 mM) and penicillin/streptomycin (200 units/ml). For all experiments, the cells were changed to serum-free conditions in Williams E medium containing the above additions, except that the foetal calf serum was replaced by BSA (0.2%, w/v).

Measurement of uptake and degradation of LDL by HepG2 cells

In experiments to assess the uptake and degradation of LDL by HepG2 cells, the cells were incubated in serum-free medium, containing the appropriate concentrations of growth factor or drug, for 18 h at 37 °C. Additions were made to the cells in sterile PBS, methanol or dimethyl sulphoxide (DMSO) (final vehicle concn. 0.5%). The cells were then washed in serum-free medium, and incubated for 5 h at 37 °C with serum-free medium containing 10 µg/ml [³H]sucrose-labelled LDL, in the absence or presence of a 30-fold excess of unlabelled LDL. The cells were placed on ice, washed three times with ice-cold PBS and solubilized in 0.5 M NaOH (1 ml). Samples (0.5 ml) were added to 5 ml of Picofluor scintillant, and the radioactivity was determined as described below. This method depends on the lysosomal trapping of sucrose (which is covalently bound to the apoprotein B moiety of LDL) following internalization and degradation of the LDL particle. The total amount of sucrose accumulating in cultured fibroblasts has been shown to correlate well with the amount of iodinated LDL taken up and degraded (Pittman et al., 1979). The radioactivity associated with the cell is therefore a measure of total LDL uptake and degradation by the cells (including the cell-surface-associated LDL) and is expressed as ng of LDL/mg of cell protein.

Measurement of cholesterol biosynthesis from [U-¹⁴C]acetate

The cholesterol biosynthesis rate was measured essentially as described by Kosykh et al. (1989). HepG2 cells were incubated with serum-free medium, containing growth factors or drugs and sodium [U-¹⁴C]acetate (2 µCi/ml, 0.04 mM) for 18 h. Cells were washed three times with PBS, before being scraped into 1 ml of PBS and sonicated at 22 kHz for 10 s to give cell homogenates. Samples (0.5 ml) of cell homogenate were extracted by the method of Bligh and Dyer (1959). An internal standard was added to the extraction buffer ([³H]cholesterol, 1.88 nCi/ml, 0.041 nM). Samples of the lipid extract were applied to silica t.l.c. plates (Whatman) and developed in hexane/diethyl ether/acetic

acid (35:15:1, by vol.). The cholesterol band was identified by co-chromatography with standard and scraped from the plate into 5 ml of Picofluor scintillant. Radioactivity was determined in a Beckman LS5801 counter and corrected for counting efficiency by the external-standard method. Cell homogenates were assayed for protein by the BCA assay (Pierce).

Measurement of cholesteryl ester formation by HepG2 cells

Incorporation of radiolabelled oleate into cholesteryl oleate was measured essentially as described by Goldstein et al. (1983), except that [9,10(n)-³H]oleate/BSA complex (79 Ci/mol) was used. A stock solution of [³H]oleate/BSA complex was prepared of 10 mM [³H]oleate complexed to 1.2 mg/ml BSA. A sample (1 µl) of this complex was then added to 1 ml of Williams E medium containing 0.2% BSA, as described above, giving a final concentration of 10 µM [³H]oleate. After incubation for 24 h, the cells were washed three times with PBS, and lipids were extracted from the cell homogenates, as described above except that cholesteryl [1-¹⁴C]oleate was added as the internal standard (0.45 nCi/ml, 8 nM). The neutral lipids were then separated on t.l.c. in light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (90:30:1, by vol.). The cholesteryl oleate band was identified by co-chromatography with standard, scraped from the plate into Picofluor scintillant and the radioactivity determined, as described above. Results are expressed as nmol of [³H]oleate incorporated/mg of protein.

Measurement of incorporation of [³H]thymidine into DNA by HepG2 cells

HepG2 cells were cultured as described above, before being changed to fresh medium containing 0.2% BSA and 1 µCi/ml [methyl-³H]thymidine (84 Ci/mol), together with the appropriate concentrations of growth factor or phorbol ester. The cells were incubated for 24 h, and then washed three times with PBS and scraped into 1 ml of PBS. The protein was precipitated with 10% TCA, and the resulting pellet was washed twice with 10% TCA. The final pellet was solubilized in 1 ml of 0.5 M NaOH and counted for radioactivity (d.p.m.). The results are expressed as pmol of [³H]thymidine incorporated/mg of cell protein.

Statistics

All experiments were performed with triplicate wells, and the numbers of independent experiments performed are indicated in the legends to the Figures and Tables. Statistical analysis was performed by the Student's *t* test. A statistically significant difference was reported when *P* < 0.05.

RESULTS

Effect of EGF and PMA on the uptake and degradation of [³H]LDL by HepG2 cells

Uptake and degradation of [³H]LDL by HepG2 cells was saturable, with half-maximal uptake at around 20 µg of LDL/ml, which agrees well with previously reported values in HepG2 cells with iodinated LDL (Havekes et al., 1983). Non-specific uptake and degradation (assessed in the presence of 30-fold excess of unlabelled LDL) represented around 10–12% of total uptake (results not shown).

The addition of EGF to HepG2 cells for 18 h stimulated LDL uptake and degradation in a concentration-dependent manner, with near-maximal stimulation achieved at 20 ng of EGF/ml (Figure 1a). The maximum stimulation achieved at 100 ng of

Figure 1 Stimulation of $[^3\text{H}]$ LDL uptake by EGF and PMA

HepG2 cells were incubated in serum-free medium with the concentrations of EGF and PMA indicated for 18 h, before assaying LDL uptake and degradation (as described in the Methods section). (a) Concentration-dependence for EGF stimulation of LDL uptake in the absence (□) or presence (○) of PMA (100 ng/ml). (b) Concentration-dependence for PMA stimulation of LDL uptake in the absence (■) or presence (●) of EGF (100 ng/ml). The Figure shows the means $\pm$ S.D. from triplicate wells in a typical experiment. The results were reproduced in a further independent experiment. Values significantly different from the control are represented by *, values significantly different from the incubation with one agent alone are represented by ** ($P < 0.05$).

EGF/ml in this experiment was 225% of the control value. In subsequent experiments (Table 1), a mean increase of 288.5% of the control value was observed at 100 ng of EGF/ml ($P < 0.0001$, $n = 11$). When comparing triplicate wells within this experiment, all concentrations of EGF significantly stimulated LDL uptake ($P < 0.05$) above the control incubation. The presence of PMA (100 ng/ml) (Figures 1a and 1b) increased LDL uptake and degradation by 305% in this experiment. In subsequent experiments (Table 1), PMA stimulated LDL uptake by 325.8% of the control value ($P < 0.0005$, $n = 7$). PMA increased uptake of LDL at all concentrations of EGF tested, in an additive manner (Figure 1a). Similarly, the presence of PMA significantly increased LDL uptake in a dose-dependent manner (Figure 1b), and this effect was further increased by the presence of EGF (100 ng/ml).

Since peptide growth factors can increase LDL receptor number by stimulating cell proliferation (Chait et al., 1980), we measured the incorporation of $[^3\text{H}]$ thymidine into DNA during an 18 h incubation in the presence of EGF (100 ng/ml) and PMA (100 ng/ml). The incorporation of $[^3\text{H}]$ thymidine (0.338 ± 0.063 pmol/mg; mean $\pm$ S.D., $n = 5$) did not vary by

Table 1 Effects of EGF, PMA, Ro-31-8220 and genistein on the uptake and degradation of $[^3\text{H}]$ LDL by HepG2 cells

HepG2 cells were preincubated for 18 h in serum-free medium containing the growth factors or drugs indicated. The uptake and degradation of $[^3\text{H}]$ sucrose-labelled LDL was then assessed as described in the Methods section. Results are expressed as ng of LDL/mg of cell protein, and are means $\pm$ S.E.M. for the numbers of independent experiments shown in parentheses. Values that differ significantly are indicated by their P values (vs., versus); NS, not significantly different.

Additions	$[^3\text{H}]$ LDL (ng/mg)	Paired t test
I None	340.8 ± 56.0 (13)	
II EGF (100 ng/ml)	983.3 ± 117.9 (11)	I vs. II $P < 0.0001$
III PMA (100 ng/ml)	1110.5 ± 178.7 (7)	I vs. III $P < 0.0005$
IV Ro-31-8220 (10 μM)	215.8 ± 50.2 (4)	I vs. IV NS
V PMA (100 ng/ml) + Ro-31-8220 (10 μM)	473.2 ± 117.3 (5)	I vs. V NS
VI EGF (100 ng/ml) + Ro-31-8220 (10 μM)	639.25 ± 79.6 (4)	III vs. V $P < 0.05$ I vs. VI $P < 0.0005$
VII EGF (100 ng/ml) + PMA (100 ng/ml)	2490.4 ± 153.1 (5)	II vs. VI NS II vs. VII $P < 0.0005$
VIII EGF (100 ng/ml) + PMA (100 ng/ml) + Ro-31-8220 (10 μM)	693.03 ± 79.1 (3)	III vs. VII $P < 0.005$ I vs. VIII $P < 0.01$ II vs. VIII NS III vs. VIII NS
IX Genistein (100 μM)	142.16 ± 83.1 (3)	VII vs. VIII $P < 0.01$ I vs. IX $P < 0.05$
X EGF (100 ng/ml) + genistein (100 μM)	255.96 ± 69.0 (5)	I vs. X NS
XI PMA (100 ng/ml) + genistein (100 μM)	943.1 ± 243.6 (6)	II vs. X $P < 0.005$ I vs. XI $P < 0.05$ III vs. XI NS

more than 10% in the presence of any of the agents tested, either singly or in combination, indicating that the increased uptake of $[^3\text{H}]$ LDL shown is not due to an increase in cell numbers.

Effect of the protein kinase C inhibitor Ro-31-8220 on uptake and degradation of $[^3\text{H}]$ LDL by HepG2 cells

Addition of the protein kinase C inhibitor Ro-31-8220 at 10 μM did not significantly affect LDL uptake and degradation by HepG2 cells (Table 1). The stimulation of LDL uptake by PMA (100 ng/ml) was decreased ($P < 0.05$) by the presence of Ro-31-8220 (10 μM) to a level not significantly different from the control value. The increase in LDL uptake and degradation due to EGF (100 ng/ml) was not significantly decreased by the presence of 10 μM Ro-31-8220. The additive effect of EGF (100 ng/ml) and PMA (100 ng/ml) together was again shown, and this effect was attenuated by the presence of Ro-31-8220 ($P < 0.01$), to a level which was not significantly different from the condition where EGF was present alone.

Effect of down-regulation of protein kinase C on EGF stimulation of LDL uptake by HepG2 cells

It is well established that chronic activation of protein kinase C results in down-regulation of the enzyme activity, due to increased proteolysis (Stabel and Parker, 1991). We therefore examined the effect of depleting HepG2 cells of protein kinase C activity, by treating the cells with PMA (200 ng/ml) for around 90 h in Williams E medium containing 10% FCS (as described previously). A parallel experiment was performed in which the cells received an equivalent volume of DMSO. The cells were then changed to serum-free conditions and stimulated with EGF

Table 2 Effect of down-regulation of protein kinase C on EGF stimulation of LDL uptake by HepG2 cells

HepG2 cells were pretreated with PMA (200 ng/ml), or with an equivalent amount of DMSO, for 90 h. The cells were then incubated in serum-free medium containing EGF (100 ng/ml), PMA (100 ng/ml), or EGF (100 ng/ml) and PMA (100 ng/ml) together. The values shown are relative to the control incubation in cells which were not treated with PMA, and are the means $\pm$ ranges of two independent experiments. The absolute values for specific uptake and degradation of [3 H]LDL in the two individual control incubations were 492.54 and 108.86 ng of LDL/mg.

Additions	Pretreatment ...	Relative uptake (%)	
		DMSO	PMA
I None		100	80.4 $\pm$ 20.6
II EGF (100 ng/ml)		278.9 $\pm$ 66.7	265.6 $\pm$ 121.4
III PMA (100 ng/ml)		154.3 $\pm$ 19.1	60.0 $\pm$ 6.2
IV EGF (100 ng/ml) + PMA (100 ng/ml)		635.1 $\pm$ 266.2	320.6 $\pm$ 49.2

(100 ng/ml), PMA (100 ng/ml) or with EGF and PMA together, before uptake and degradation of [3 H]LDL was assessed exactly as described above. LDL uptake was not altered in the cells pretreated with PMA, compared with the control incubation in both of the experiments performed (Table 2). The stimulation of LDL uptake and degradation by PMA (100 ng/ml) in the control cells was not as great as observed previously (in two experiments the mean increase was $154.3 \pm 19.1\%$), and this decrease in stimulation may be due to the increased confluency of the cells. However, in the PMA-pretreated cells, when challenged again with PMA, there was a decrease in the uptake and degradation of LDL to $60 \pm 6.1\%$ of the control value. The increase in uptake and degradation of LDL by HepG2 cells due to EGF (100 ng/ml) was unimpaired by pretreatment with PMA, indicating that this response was independent of protein kinase C. In the presence of both EGF and PMA together, the previously described increased uptake and degradation of LDL was observed in the control cells. In the pretreated cells, however, the increased uptake was not observed and the response was equivalent to the condition where EGF was present alone. These results indicate that stimulation of LDL uptake by EGF can occur independently of protein kinase C activation.

Effect of the tyrosine kinase inhibitor genistein on uptake and degradation of [3 H]LDL by HepG2 cells

The addition of genistein (100 μ M) to HepG2 cells resulted in a decrease in LDL uptake to around 41% of the control value ($P < 0.05$) (Table 1). In general, the responses observed in the presence of genistein were more variable than in the absence of this compound. This may be because genistein has been reported to cause a number of cytotoxic effects when used at the concentrations required to prevent EGF-receptor tyrosine phosphorylation (Akiyama and Ogawara, 1991). However, the cells were still able to respond by increasing LDL uptake when stimulated by PMA (100 ng/ml). The stimulation of LDL uptake by EGF (100 ng/ml) was reversed by the presence of genistein to a level which was not significantly different from the control incubation. This is consistent with the fact that the first step in the EGF cell-signalling pathway involves the activation of the receptor tyrosine kinase (Carpenter and Cohen, 1990).

Figure 2 Effect of EGF and PMA on the esterification of cholesterol by HepG2 cells

HepG2 cells were preincubated for 18 h in serum-free medium containing the appropriate concentrations of EGF ($\circ$, $\bullet$) or EGF in the presence of PMA ($\square$, $\blacksquare$) (100 ng/ml). The cells were then incubated with fresh serum-free medium containing [3 H]oleate in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 200 μ g LDL/ml for a further 24 h. The results shown are relative to the control incubation in the presence of LDL (200 μ g/ml) and are means $\pm$ S.E.M. for three independent experiments. In the presence of LDL, each concentration of EGF gave significantly increased rates of esterification ($P < 0.05$). The presence of EGF and PMA further stimulated esterification rates significantly compared with EGF alone ($P < 0.05$) in the presence of LDL. The absolute value for the control incubation was 0.412 ± 0.134 nmol of [3 H]oleate incorporated/mg of cell protein (mean $\pm$ S.E.M., $n = 3$).

Effect of EGF and PMA on the esterification of cholesterol by HepG2 cells

To examine whether the increased uptake and degradation of [3 H]LDL was associated with an increased flux of cholesterol into the cell, we investigated the incorporation of [3 H]oleate into cholesteryl ester by HepG2 cells. Cells were preincubated with increasing concentrations of EGF, in the absence or presence of 100 ng/ml PMA, for 18 h. The cells were then incubated with fresh medium containing [3 H]oleate (as described in the Methods section) in the absence or presence of 200 μ g of LDL/ml of medium, for a further 24 h. The incorporation in the absence of LDL indicates the direct effect of these agents on cholesterol esterification; the incorporation in the presence of LDL indicates the effect due to enhanced LDL uptake and degradation in these cells. In the absence of LDL (Figure 2), the incorporation of [3 H]oleate into cholesteryl ester represented around 60% of the control value assessed in the presence of LDL. There was no direct effect of EGF or PMA on cholesterol esterification. In the presence of LDL, preincubation with EGF increased the level of esterification in a concentration-dependent manner. The stimulatory effect was near maximal by around 20 ng of EGF/ml, reaching 175% of the control value at 100 ng of EGF/ml ($P < 0.005$). The presence of PMA in the preincubation increased the subsequent esterification, in the presence of LDL, by around 250% of the control value ($P < 0.01$). PMA in combination with EGF significantly increased esterification compared with the condition where EGF was present alone. When compared with the data from Figure 1, the relative increases in uptake of LDL were highly correlated with the relative increases in cholesterol esterification ($r = 0.965$, $r^2 = 0.931$). The results confirm that the increased uptake of LDL described above does result in an increased flux of cholesterol into the cells, and that the effects of EGF and PMA persist even when these agents are removed from the cells for a further 24 h.

Table 3 Effect of EGF and PMA on synthesis of cholesterol from [14 C]acetate by HepG2 cells

HepG2 cells were incubated in serum-free medium containing the concentrations of growth factor or drug indicated and 2 μ Ci/ml [14 C]acetate. After 18 h, the incorporation of [14 C]acetate into cholesterol was assessed as described in the Methods section. Results are means $\pm$ S.E.M. from three independent experiments, and are expressed as nmol of [14 C]acetate incorporated/mg of cell protein. Values that differ significantly from the control incubation ($P < 0.05$) are indicated *.

Additions	Cholesterol synthesis (nmol of [14 C]acetate/mg)
I None	0.357 $\pm$ 0.066
II EGF (100 ng/ml)	0.372 $\pm$ 0.046
III PMA (200 ng/ml)	0.593 $\pm$ 0.067*
IV EGF (100 ng/ml) + PMA (200 ng/ml)	0.593 $\pm$ 0.086*
V EGF (100 ng/ml) + Ro-31-8220 (10 μ M)	0.249 $\pm$ 0.037
VI PMA (200 ng/ml) + Ro-31-8220 (10 μ M)	0.289 $\pm$ 0.048
VII 25-Hydroxycholesterol (1 μ M)	0.204 $\pm$ 0.026*

Table 4 Effect of mevinolin (1 μ M) on EGF stimulation of LDL uptake by HepG2 cells

HepG2 cells were incubated for 18 h in serum-free medium containing EGF (100 ng/ml), mevinolin (1 μ M) or EGF (100 ng/ml) and mevinolin (1 μ M) together. The control incubation contained the appropriate volume of vehicle. [3 H]LDL uptake was then assessed as described in the Methods section. Results are the means $\pm$ ranges for two independent experiments.

Additions	[3 H]LDL (ng/mg)
I None	164.0 $\pm$ 0.91
II EGF (100 ng/ml)	455.2 $\pm$ 7.54
III Mevinolin (1 μ M)	281.5 $\pm$ 6.56
IV EGF (100 ng/ml) + mevinolin (1 μ M)	867.3 $\pm$ 47.23

Effect of EGF and PMA on synthesis of cholesterol from [14 C]acetate in HepG2 cells

We investigated whether the changes described for LDL uptake were reflected in the rate of cholesterol synthesis (Table 3). EGF (100 ng/ml) did not significantly affect the incorporation of [14 C]acetate into cholesterol during the 18 h incubation period. PMA (100 ng/ml) significantly increased cholesterol synthesis to around 170% of the control value ($P < 0.05$). The addition of EGF and PMA together did not further increase cholesterol synthesis from [14 C]acetate. The effect of PMA was reversed by the addition of Ro-31-8220 (10 μ M). Ro-31-8220 also appeared to decrease the rate of cholesterol synthesis in the incubation with EGF, but this decrease was not significant in the three experiments performed. In order to demonstrate the sterol control of cholesterol synthesis, 25-hydroxycholesterol (1 μ M) was included in all three experiments. This oxysterol decreased cholesterol synthesis to around 57% of the control value ($P < 0.05$).

Effect of inhibition of cholesterol synthesis on stimulation of LDL uptake by EGF

In order to demonstrate further that the stimulation of LDL uptake and degradation by EGF occurs independently of changes

in cholesterol biosynthesis rate, we examined the effect of the HMG-CoA reductase inhibitor mevinolin, in combination with EGF (Table 4). Mevinolin (1 μ M) increased the uptake and degradation of LDL to 171 $\pm$ 5.6% in the two independent experiments performed. EGF (100 ng/ml) again stimulated uptake and degradation of LDL, and in combination with mevinolin (1 μ M) increased LDL uptake and degradation to 528 $\pm$ 25.8% of the control value.

DISCUSSION

Activation of EGF receptors elicits multiple intracellular signals after activation of the receptor tyrosine kinase, including activation of phospholipase C- γ , resulting in phosphoinositide turnover, release of diacylglycerol and activation of protein kinase C (Carpenter and Cohen, 1990). EGF induces a rapid Ca^{2+} influx in hepatocytes (Mine et al., 1991) and has been reported to activate a phospholipase A_2 activity (Spaargaren et al., 1992), which may be involved in Ca^{2+} -channel activation in some cell types (Wright et al., 1992). The cell signalling events which lead to the EGF stimulation of LDL uptake by HepG2 cells may involve one, or a combination, of these pathways. In this study, we have examined the role of protein kinase C, and of tyrosine kinases, in the sterol-independent activation of LDL uptake by EGF in HepG2 cells.

The down-regulation of protein kinase C activity by prolonged treatment with PMA abolished the response of the pretreated HepG2 cells to PMA, on restimulation. In these pretreated cells, EGF was able to stimulate the uptake and degradation of LDL to the same extent as in the PMA-responsive cells. This indicated that the activation of protein kinase C was not necessary for the EGF-induced increase in LDL uptake in HepG2 cells. However, it has been reported that prolonged treatment with phorbol esters can result in effects which are not mediated by protein kinase C, but which can be attributed to membrane perturbations (Tao et al., 1989) or to antagonistic effects on Ca^{2+} channels (Hockberger et al., 1989). We therefore utilized the specific protein kinase C inhibitor Ro-31-8220 (Davis et al., 1992). This compound is highly selective for protein kinase C over protein kinase A (Davis et al., 1992), Ca^{2+} /calmodulin-dependent kinase (Davis et al., 1989) and phosphorylase kinase (Elliott et al., 1990). The compound has been shown to block the PMA-induced activation of phospholipase D in human neutrophils with an IC_{50} of 0.3 μ M (Uings et al., 1992), and to inhibit protein kinase C phosphorylation completely in human platelets at 3 μ M (Walker and Watson, 1993). In our experiments we found that a concentration of 10 μ M Ro-31-8220 was required to block completely the effects of PMA. Under these conditions, EGF stimulation of LDL uptake was unaffected by the inhibition of protein kinase C. Furthermore, the additive effects on uptake observed in the presence of EGF and PMA together suggest that the pathways stimulated by these agents are not identical. These results provide strong evidence to suggest that protein kinase C activation is not required for EGF stimulation of LDL uptake in HepG2 cells.

Genistein has been shown to inhibit tyrosine kinases in a number of cell types (Akiyama and Ogawara, 1991) and is relatively inactive against serine/threonine protein kinases (Akiyama et al., 1987). In A431 cells, genistein inhibits the EGF-stimulated increase in cell phosphotyrosine levels with an IC_{50} of 111 μ M (Akiyama et al., 1987). Genistein blocks the effects of PDGF (tyrosine phosphorylation, induction of early response genes, DNA synthesis) (Hill et al., 1990), although it has also been shown to exert some non-tyrosine kinase-dependent effects (Akiyama and Ogawara, 1991; Huang et al., 1992). EGF elicits

increased levels of phosphotyrosine in HepG2 cells (Grove et al., 1992). The EGF stimulation of LDL uptake was blocked by the presence of genistein, and this is consistent with the action of the EGF-receptor tyrosine kinase as an obligatory first event in the cell-signalling pathway (Chen et al., 1987). Stimulation of LDL uptake by PMA was not affected by genistein, suggesting that tyrosine kinases are not involved in the protein kinase C stimulation of LDL uptake.

It is therefore apparent that EGF can activate LDL uptake in HepG2 cells by a cell signalling pathway which appears to involve tyrosine kinases, but not protein kinase C. Oncostatin M, a macrophage-derived growth factor, has been shown to increase LDL receptors in HepG2 cells (Grove et al., 1992), but by a cell signalling pathway which involves both tyrosine kinases and protein kinase C. PDGF, which stimulates LDL receptor expression in smooth-muscle cells (Chait et al., 1980) by increasing the expression of the LDL receptor gene, can do so independently of protein kinase C (Roth et al., 1991). It has also recently been shown by Kamps and van Berkel (1993) that, although PMA stimulates LDL receptor expression in HepG2 cells, it does not do so in primary hepatocytes from humans or rats. Distinct cell signalling pathways, stimulating LDL receptor expression, therefore exist for different growth-regulatory polypeptides, and these pathways can differ, depending on cell type.

It is also evident that the increases in LDL uptake observed in the presence of EGF are independent of changes in cholesterol biosynthesis within HepG2 cells. There was no apparent increase in cholesterol biosynthesis during the 18 h incubation with EGF, and inhibition of cholesterol biosynthesis with the HMG-CoA reductase inhibitor mevinolin also did not prevent the stimulation of LDL uptake by EGF. Furthermore, treatment with EGF did not increase incorporation of thymidine into DNA, indicating that the increased LDL uptake, at least over 18–24 h, occurs in the absence of cell proliferation. This is in contrast with the situation in human skin fibroblasts and smooth-muscle cells, where induction of LDL receptor activity by insulin (Chait et al., 1979) or by PDGF (Chait et al., 1980) was accompanied by stimulation of endogenous cholesterol synthesis and increased incorporation of thymidine into DNA (Chait et al., 1980; Davies and Kerr, 1982; Witte et al., 1982). In HepG2 cells, however, oncostatin M stimulates LDL receptor expression without a corresponding rise in cholesterol synthesis or incorporation of thymidine into DNA (Grove et al., 1992). Ellsworth et al. (1991) have also reported the presence of a serum factor which mediates induction of LDL receptors without increasing cholesterol biosynthesis.

Therefore, the pathway by which EGF increases uptake and degradation of LDL by HepG2 cells would, if found in human liver, represent a cholesterol-independent mechanism by which circulating levels of cholesterol could be lowered. Further experiments are required to identify the components of the cell signalling pathway responsible for EGF stimulation of LDL uptake in the liver, which may be distinct from those operating in other cell types.

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