Effects of Platelet-Derived Growth Factor on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Cultured Human Fibroblasts

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Studies were conducted to delineate in detail the effects of platelet-derived growth factor (PDGF) upon 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity in cultured human fibroblasts. PDGF has a variety of coordinated effects on low density lipoprotein (LDL) and cholesterol metabolism and on DNA synthesis in these cells. We have previously shown that there is a critical time period, 10 to 20 hours after PDGF addition to quiescent cells, when mevalonate (MVA) is required for DNA synthesis to occur. The present studies demonstrate that PDGF produces a biphasic stimulation of reductase activity in cultured fibroblasts: a peak at 4 to 6 hours, followed by a decline, and then a second smaller peak at 24 hours, concurrent with DNA synthesis. The stimulation of both peaks was PDGF concentration-dependent, although quantitative differences were observed. Proportionally similar, LDL concentration-dependent reductions in both peaks of reductase activity were also seen. PDGF stimulated cholesterol synthesis from acetate in intact cells reaching peak values after 24 to 28 hours. The two peaks of reductase activity stimulated by PDGF neither coincide with the critical time period when mevalonate is needed for later DNA synthesis to occur, nor reflect the pattern of cholesterol synthesis within the cells. (Arteriosclerosis 6:34–41, January/February 1986)

It is well-known that the proliferative capacity of cells in response to mitogenic stimuli depends in part on mechanisms that enable the cells to obtain an increased supply of cholesterol.1–3 This cholesterol can be provided in the medium in the form of lipoproteins, or synthesized endogenously. The inclusion of lipoproteins as low density lipoproteins (LDL) in the medium of cultured cells suppresses endogenous sterol synthesis mainly through an effect on the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (EC 1.1.1.34).4–6 Cholesterol esterification is concomitantly stimulated by an increased activity of acyl CoA-cholesterol acyltransferase.7

We have used human fibroblasts stimulated with platelet-derived growth factor (PDGF) to study changes in cholesterol metabolism that occur in growing cells. Stimulation of cells with PDGF caused a dose-related increase in 125I-LDL binding that paralleled the PDGF stimulation of DNA synthesis and cell growth.8–10 This effect was shown to be due to an approximately fourfold increase in the number of receptor sites per cell rather than to changes in receptor affinity.10 Increases in internalization and degradation of the bound 125I-LDL paralleled the increase in binding.10 When LDL was not available to PDGF-stimulated cells, there was a dose-related increase in HMG CoA reductase activity of approximately fourfold.10

Several recent studies have suggested that both cholesterol and a product of mevalonate (MVA) metabolism other than cholesterol may be necessary for DNA synthesis and cell growth.11–19 These studies have used compactin18 or mevinolin,10 potent competitive inhibitors of HMG CoA reductase. When MVA synthesis is markedly blocked by these compounds, cell growth is also inhibited. Restoration of
cell growth could not be achieved by the addition of lipoproteins or cholesterol but required the presence of MVA. We have recently reported that in human fibroblasts stimulated with PDGF, there is a critical time period, several hours before S phase, when the cells require MVA in order for DNA synthesis to proceed at 24 hours. These observations raised questions concerning the relationships that exist between the effects of PDGF on DNA synthesis, and on the activity of HMG CoA reductase. The studies reported here were designed to explore these relationships in detail.

**Methods**

**Materials**

The 2-14C-acetic acid (55 mCi/mmol), methyl-3H-thymidine (2 Ci/mmol), RS-5-3H(N)-mevalonolactone (13.8 Ci/mmol), 3H-amino acids (57 mCi/mmol) and 1,2-3H-cholesterol (40.7 Ci/mmol) were purchased from New England Nuclear (Boston, Massachusetts). Cholesterol and sodium acetate were purchased from Applied Science, Foster City, California. Calf serum (CS) was obtained from Sterol Systems, Logan, Utah. Dulbecco’s modified Eagle’s (DME) medium, penicillin (10,000 μg/ml), and streptomycin (10,000 μg/ml) were purchased from Gibco, Grand Island, New York. Kyro EOB was kindly provided by Proctor and Gamble, Cincinnati, Ohio.

**Preparation of Low Density Lipoprotein and Plasma-Derived Lipoprotein-Deficient Serum**

Human LDL (density = 1.02–1.05 g/ml) was obtained from fresh human plasma by differential ultracentrifugation. Human plasma-derived lipoprotein-deficient serum (PDLDS) was prepared from fresh plasma as previously described. Preparations of partially purified platelet-derived growth factor (PDGF) were prepared from 50-unit batches of outdated human platelet-rich plasma as previously reported. The resulting preparations were estimated to contain PDGF purified approximately 20 to 30 × 10³-fold.

**Cell Culture**

The normal human fibroblasts (N-1) used in these experiments were established in our laboratory as described previously. The homozygous familial hypercholesterolemic (FH) cells used (GM203) were obtained from the Genetic Mutant Cell Repository in Camden, New Jersey. Stock culture medium contained 10% calf serum (vol/vol) in DME medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). Cells were cultured as described previously with a 48-hour period in 5% PDLDS from Day 6 to Day 8 after plating. Experiments were begun on Day 8 at a cell density of 5–6 × 10⁵ cells/35 mm dish.

**DNA Synthesis**

Incorporation of ³H-thymidine into DNA during a 30-minute period was used to measure the rate of DNA synthesis, as described previously. Cells were made quiescent by 48-hour incubation in 5% PDLDS in DME medium. Addition of a maximum stimulatory dose of partially purified PDGF (approximately 2 μg/ml) then initiated the passage of the cells through the cell cycle, with the peak of DNA synthesis occurring 24 hours after addition of PDGF.

**Acetate Incorporation into Cholesterol**

Incorporation of ¹⁴C-acetate into ¹⁴C-labeled non-saponifiable lipids was measured according to a modification of the procedure of Faust et al., as described previously. Cells were incubated with 2-¹⁴C acetate (10 μCi and 1 μmol/plate) for 2 hours at 37° C. Cells were harvested, pooled with the medium, saponified for 3 hours at 70° C, and extracted three times with petroleum ether (90% efficiency). Extracts were evaporated, and the material resuspended and spotted on silica Gel G plates (Brinkmann, Westbury, New York). Thin-layer chromatography was conducted with CHCl₃ as solvent, and standard spots of cholesterol were run on the same plates. The cholesterol spots were scraped from the plates, counted by liquid scintillation, and quantified with the use of an internal standard of ³H-cholesterol.

**Hydroxymethylglutaryl Coenzyme A Reductase Assay**

Enzyme activity was assayed according to the method of Alberts et al., with modifications. The assay measured the amount of product ⁴C-mevalonate formed from added labeled substrate ⁴C-HMG CoA. Product is separated from unreacted substrate by conversion of MVA to the lactone form, followed by ion-exchange chromatography. Conditions were selected to provide a linear reaction rate with increasing enzyme (cell extract) added during a 60-minute incubation time. Cells were harvested and washed in Buffer A (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4), and frozen in liquid N₂, after pelleting of cells by centrifugation and removal of all traces of Buffer A by suction. At the time of assay, samples were thawed, 200 μl of freshly-made Buffer B (50 mM potassium phosphate, 5 mM dithiothreitol (DTT), 5 mM EDTA, 0.2 M KCl and 0.25% Kyro EOB, pH 7.4) was added to each tube, and samples were incubated at 37° C for 20 minutes. After incubation, samples were spun for 1 minute at 12,000 rpm, and 75 μl of each sample supernatant was added to an assay tube containing 10 μl of 0.1 M DTT, 5 μl 1 M potassium phosphate buffer, and 5 μl of 0.1 M NADPH.
HOURS AFTER TRANSFER FROM
10% CS TO 5% PDLDS

Figure 1. Rise in HMG CoA reductase activity during the time that cells are becoming quiescent in 5% plasma-derived lipoprotein-deficient serum (PDLDS). This graph shows the results of two separate experiments that used N-1 cells. Cells were plated on Day 0 at 3 × 10^4 cells/dish. On Day 6, the medium was changed from 10% calf serum (CS) to 5% PDLDS. At various time intervals thereafter, triplicate plates were harvested and assayed for HMG CoA reductase activity. The means (± 1 SD) of these triplicate assays are shown.

(dissolved in 0.05 M Tris at pH 9); the final pH of the solution mixture was 7.4. Assay tubes were brought to 37° C in a water bath, and 5 μl of 14C-HMG CoA was then added to each tube (10 μCi/2.5 μmol/ml). After a 1-hour incubation, the reaction was stopped with the addition of 20 μl 5 N HCl. After a 10-minute incubation to ensure lactonization, 10 μl 3H-mevalonolactone solution (1 × 10^6 cpm) was added to each tube as an internal standard. Samples were then subjected to ion exchange chromatography on a small column (1 × 5 cm) of Bio Rex-5 resin (100–200 mesh). The reaction product, mevalonolactone, was separated from substrate by washing with H2O, and the eluate was collected and assayed for radioactivity.

Other Methods
Protein concentrations were measured by the method of Lowry et al.23 by using a protein standard solution from Sigma. Cell numbers were determined with a Coulter Counter, Model Z, or a hemacytometer. Radioassay for 14C and 3H was carried out in a Model 3255 Packard liquid scintillation counter. Results in the figures are presented as means ± 1 SD.

Results
Rise in Hydroxymethylglutaryl Coenzyme A Reductase Activity during Quiescence

Figure 1 shows the time course of the change in HMG CoA reductase activity during the period when cells ceased to grow and became quiescent. After transfer to medium lacking both lipoproteins and platelet proteins (particularly PDGF), the enzyme activity rose gradually for 1 to 3 days thereafter. The rate of MVA production per 10^6 cells during the late log phase of growth in 10% calf serum was 0.5 to 1.0 pmol/minute. The fibroblasts spent the next 2 days in medium from which both platelet proteins and lipoproteins had been removed (5% PDLDS), and during that time MVA production rose at least fourfold to 4.0 ± 0.9 (mean ± SD, n = 9) pmol/minute.

Increases in Hydroxymethylglutaryl Coenzyme A Reductase Stimulated by Platelet-Derived Growth Factor

Figure 2 shows the effects of platelet-derived growth factor (PDGF) on HMG CoA reductase activity in normal human fibroblasts. Quiescent cell monolayers were prepared as described in Methods. PDGF (partially purified) was added in increasing concentrations, and the plates were harvested in triplicate for assay of HMG CoA reductase activity at 5 hours and 24 hours after PDGF addition. Mean (±1 SD) values are shown.
Figure 3. Time course of the effects of platelet-derived growth factor (PDGF) on HMG CoA reductase activity, DNA synthesis, and total protein synthesis in normal human fibroblasts. Quiescent cell monolayers were prepared, and PDGF was added 8 days later. At various time intervals thereafter, one set of plates (in triplicate) was used for the determination of each of the three parameters: 1) a 15-minute pulse of ³H-thymidine for determination of DNA synthesis (●); 2) assay of HMG CoA reductase (○); and 3) a 5-minute pulse of ³H-amino acids for determination of total protein synthesis (△).

After the addition of PDGF, reductase activity increased to reach a maximum of more than threefold greater than baseline at approximately 4 to 6 hours. This rise roughly coincided in time with an initial increase in total protein synthesis that also occurred about 4 hours after the PDGF addition (Figure 3). HMG CoA reductase activity then declined progressively until 18 hours, followed by a second increase in enzyme activity that resulted in an approximate doubling of HMG CoA reductase activity at 24 hours, concurrent with the peak of DNA synthesis. Thus, the time course of the rise in HMG CoA reductase activity produced by the PDGF addition was characteristically biphasic. In a dozen separate experiments that used both FH and normal cell lines, an average threefold increase occurred at 4 to 6 hours, and an average 1.4-fold increase occurred at 24 hours.

**Effects of Low Density Lipoprotein on Hydroxymethylglutaryl Coenzyme A Reductase**

The level of reductase activity in cells is related directly to the availability of LDL in the medium. A detailed study was conducted to determine the effects of increasing concentrations of LDL on the time course of reductase stimulation by PDGF. To conduct these studies using LDL, we first had to determine the amount of LDL required to completely suppress HMG CoA reductase in nonstimulated cells and the length of time required to achieve this suppression. Figure 4 A shows the concentration-dependent effects of LDL on HMG CoA reductase activity in the absence of PDGF. A maximal decrease in HMG CoA reductase activity in the fibroblasts used in these studies was observed at a level of 100 to 200 μg LDL protein/ml. As seen in Figure 4 B, the addition of LDL (200 μg protein/ml) caused the enzyme activity to fall rapidly to a 50% decrease in 3 hours and a 90% decrease in 8 hours; the activity reached a plateau of approximately 0.2 pmol/min/10⁶ cells by 12 to 24 hours after the LDL addition.

We then explored the time course of the effects of increasing levels of LDL (0–200 μg/ml) on HMG CoA reductase activity in PDGF-stimulated cells. As shown in Figure 5, increasing concentrations of LDL produced progressive decreases in HMG CoA reductase activity throughout the entire time period studied. Enzyme activity was decreased to a roughly similar extent at each of the two time periods of peak
Figure 5. Time course of the effects of increasing concentrations of LDL on HMG CoA reductase in PDGF-stimulated normal human fibroblasts. PDGF was added to all quiescent cells at 0 time, in addition to LDL at five different concentrations (2, 6, 20, 100, and 200 μg protein/ml). The symbols used to represent HMG CoA reductase activity at various levels of LDL (measured in triplicate) are as follows: 200 μg/ml (A), 100 μg/ml (△), 20 μg/ml (●), 6 μg/ml (○), 2 μg/ml (●), and 0 μg/ml (○).

HMG CoA reductase activity. As a consequence, as shown in Figure 5, increasing levels of LDL resulted in a family of approximately parallel curves throughout the entire time period (24 hours) of PDGF stimulation.

To further investigate the time course of the effects of PDGF on HMG CoA reductase activity in the presence of LDL, we carried out a more detailed time course study of the effects of PDGF in the presence of only a very high level of LDL (200 μg/ml). The level of LDL used in this experiment corresponded to the highest level of LDL used in the experiment shown in Figure 5. Our purpose was to determine whether the length of exposure of the cells to LDL would affect the biphasic HMG CoA reductase response to PDGF. After exposure to LDL, a distinct decrease in the rate of LDL internalization is not seen before about 30 hours, since the half-time of the LDL receptor is approximately 25 hours. However, after exposure of cells to LDL, HMG CoA reductase activity decreased rapidly, with a half-time of about 3 hours, from the value present at the end of the quiescent period to a low baseline value at about 12 hours (see Figure 4B). The mechanism responsible for this very rapid decline after the addition of LDL (Figure 4B) is not defined. It was, however, of interest to investigate whether the state of down-regulation of the LDL receptor would affect the level of reductase activity during the 4- to 6-hour time period after PDGF addition.

As shown in Figure 6, the addition of PDGF produced statistically significant and quantitatively similar increments in HMG CoA reductase activity in cells that had been exposed to LDL for either 12 or 48 hours. In both instances, both peaks of enzyme activity were seen. This experiment (Figure 6) indicated that the number of LDL receptors functionally available on the fibroblasts at the time of PDGF stimulation did not influence the biphasic response of HMG CoA reductase activity to PDGF. Moreover, it clearly showed that even at the highest level of LDL used, enzyme activity was not completely suppressed.

Effects of Platelet-Derived Growth Factor on Cholesterol Synthesis

To examine the effects of PDGF on the rate and pathway of cholesterol biosynthesis, we studied the incorporation of acetate into cholesterol. Our aim was to explore whether changes in the extent of incorporation of acetate into cholesterol would parallel changes in the level of HMG CoA reductase activity produced by PDGF stimulation. When LDL was not available in the medium, the addition of 1 μg/ml PDGF increased the incorporation of 14C-acetate into 14C-esters by approximately fivefold in a 2-hour period between 22 and 24 hours (Table 1, right-hand column). As expected, when LDL was added to the medium, PDGF-stimulated fibroblasts showed much less of an increase in the amount of acetate incorporated into cholesterol. There was, however, an increase in the incorporation of acetate into a non-saponifiable lipid fraction which migrated with squalene on TLC (data not shown). This occurred in quiescent as well as in stimulated fibroblasts. Future
Table 1. Incorporation of 14C-Acetate into 14C-Cholesterol in Quiescent Fibroblasts and Fibroblasts Stimulated with Two Concentrations of Plasma-Derived Growth Factor

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Cholesterol synthesis from acetate (pmol/10^6 cells)</th>
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<tbody>
<tr>
<td></td>
<td>+ LDL</td>
</tr>
<tr>
<td>- PDGF</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>+ PDGF (250 ng/ml)</td>
<td>125 ± 14</td>
</tr>
<tr>
<td>+ PDGF (1 µg/ml)</td>
<td>165 ± 30</td>
</tr>
</tbody>
</table>

Quiescent cell monolayers were prepared as described in Methods. Platelet-derived growth factor (PDGF) was added at zero time to two concentrations (250 ng/ml and 1 µg/ml) to 35 mm plates. At 22 hours after the addition of PDGF and LDL, 14C-acetate was added to the plates for a 2-hour incubation. Each value represents the average of six plates ± 1 S. The data are expressed as the pmol of labeled acetate converted into labeled cholesterol per 10^6 cells.

Figure 7. Time course of the effects of platelet-derived growth factor (PDGF) on cholesterol synthesis from 14C-acetate in normal human fibroblasts. Quiescent cell monolayers were prepared and PDGF added at 0 time. The amount of 14C-acetate incorporated into cholesterol in a 4-hour incubation period (nmol labeled acetate/10^6 cells ± 1 S) is shown for various 4-hour intervals (with addition of 2.3 x 10^6 dpm/plate, 1 µmol/plate). The yield of 14C-cholesterol was determined as described in Methods.

Discussion

These studies were undertaken to explore in detail the effects of PDGF upon HMG CoA reductase activity in cultured human fibroblasts. Previous studies from this laboratory have shown that PDGF stimulates parallel, concentration-dependent increases in LDL binding, internalization, and degradation, and in DNA synthesis. Subsequently, we demonstrated that there is a critical time period, 10 to 20 hours after addition of PDGF, when mitogenically stimulated human fibroblasts require MVA in order for DNA synthesis to proceed at 24 hours. Since HMG CoA reductase is the enzyme responsible for MVA production and MVA is necessary for cell growth, several important questions arise. What is the time course of response of HMG CoA reductase to PDGF stimulation? How does the addition of LDL effect this enzyme activity and its response to PDGF? When is cholesterol synthesized in response to PDGF stimulation?

As reported here, PDGF produces a biphasic stimulation of HMG CoA reductase activity in human fibroblasts. A peak of activity was seen 4 to 6 hours after PDGF addition, followed by a progressive decline in reductase activity, and then a second smaller rise at 24 hours, coincident with DNA synthesis. The extent of stimulation of both peaks of reductase activity, and of DNA synthesis, was PDGF concentration-dependent over a range of PDGF levels (50 to 300 ng/ml). A major difference between the two peaks of reductase activity was observed above 0.3 µg PDGF/ml, with the early peak continuing to increase and the later peak showing no further rise.

The mechanism responsible for the observed changes in HMG CoA reductase activity after PDGF addition is not known. HMG CoA reductase is a highly regulated enzyme, with its rate reflected both by the amount and activity state of the enzyme protein. The amount of enzyme present is controlled by both its rate of synthesis and its rate of degradation, with cholesterol and other isoprenoid products of MVA exerting a negative feedback regulation on the transcription of reductase mRNA. The activity can be modulated by reversible phosphorylation-dephosphorylation of both the reductase and its kinase. The biphasic increase in reductase activity in PDGF-stimulated cells may have reflected either increases in the synthesis and amount of the enzyme or changes in its state of phosphorylation. Although definitive data on this point are not available, it seems likely that the effects observed mainly reflected
changes in the amount of enzyme. Preliminary experiments that we carried out using both NaF (an inhibitor of dephosphorylation) and alkaline phosphatase (to maximally activate the enzyme) showed no change in reductase activity (data not shown). Furthermore, Brown et al.29 have reported that all of the HMG CoA reductase in fibroblasts is in the active form under all conditions.

The second question addressed the issue of whether the two peaks of PDGF-stimulated reductase activity were differentially regulated by the presence of LDL in the medium. Proportionally similar concentration-dependent reductions in both peaks of reductase activity were seen on addition of LDL over the concentration range used (2 to 200 µg protein/ml). Since a differential effect did not occur, this suggests that a similar mechanism for enzyme regulation may exist at the two time periods. Even at the highest level of LDL used (Figure 6), PDGF still produced a distinct biphasic stimulation of reductase activity. Furthermore, very similar results were obtained (Figure 6) with cells that had been down-regulated to different degrees (exposure to LDL for either 48 or 12 hours), which suggests that the effects of PDGF on reductase activity act via processes that are at least in part independent of the processes involved in the effects of LDL on this enzyme.

Interestingly, the two peaks of PDGF-stimulated reductase activity occur at times when MVA does not appear to be needed for the cells to progress through the cell cycle. Our previous studies19 showed that if reduction of cholesterol synthesis from 14C-cholesterol appears to be needed for the cells to progress through the cell cycle. Our previous studies19 showed that if reduction of cholesterol synthesis from 14C-cholesterol obtained from our study of the time course of the synthesis rate (and of MVA synthesis) that exists within the intact cell.

In conclusion, these studies show that PDGF stimulated a biphasic increase in HMG CoA reductase activity in human fibroblasts, both in the absence and in the presence of varying amounts of LDL. Neither of the two peaks of reductase activity stimulated by PDGF coincided with the critical time period (10 to 20 hours after PDGF) when MVA was needed for later DNA synthesis. Moreover, the pattern of cholesterol synthesis within the cells also failed to reflect the biphasic pattern of reductase activity. These findings suggest that the observed stimulation of reductase activity by PDGF may not be related to the cell's need for MVA for cell growth, and/or may actually not be expressed within the intact cell.

References


Index Terms: platelet-derived growth factor • hydroxymethylglutaryl coenzyme A reductase • mevalonate • cholesterol synthesis • mitogenesis • LDL receptor pathway
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