The Effect of Three Serum Basic Proteins on the Mass of Lipids in Normal and HyperapoB Fibroblasts

Peter O. Kwiterovich, Jr, Mahnaz Motevalli, Michael Miller

Abstract We studied whether serum basic proteins (BPs) produce abnormal changes in the mass of cellular lipids in fibroblasts from patients with hyperapolipoproteinemia (hyperapoB) and if inhibition or stimulation of protein kinase C affects these processes. In normal cells, BP I increased the mean mass of triglycerides about twofold, whereas there was significantly less stimulation in hyperapoB cells (P < .005). The increase in the mass of cell cholesteryl esters seen in normal cells with BP I was also significantly reduced in hyperapoB cells (P < .005). In contrast, BP II abnormally stimulated the mass of cell cholesteryl esters sixfold in hyperapoB cells (P < .005). BP I also stimulated the mass of total phospholipids about twofold in normal cells, an effect that was reduced by about one third in hyperapoB cells (P = .08). No abnormality was found in hyperapoB cells with BP III. H-7, an inhibitor of protein kinase C, decreased the effects of BP I and BP II in normal and hyperapoB cells. C:8, an analogue of diacylglycerol, activated protein kinase C and stimulated triglyceride formation in normal (fourfold) and hyperapoB (fivefold) cells in the absence of BP I. When added with C:8, BP I further increased triglyceride production 1.5-fold in normal cells but not in hyperapoB cells. Two cellular abnormalities in lipid metabolism in hyperapoB fibroblasts were found, one with BP I, another with BP II. Protein kinase C activity was not deficient in hyperapoB cells, and the defect(s) may occur at another, perhaps earlier, step in the pathway. (Arterioscler Thromb. 1994;14:1-7.)

Key Words • triglycerides • cholesterol • cholesteryl esters • phospholipids • free fatty acids • hyperlipidemia • protein kinase C

Hyperapolipoproteinemia (hyperapoB) is a lipoprotein disorder characterized by increased numbers of small, dense low-density lipoprotein (LDL) particles. HyperapoB is prevalent in patients with coronary atherosclerosis, and an association is observed when hypertriglyceridemia is also present. HyperapoB can be accompanied by diabetes mellitus, hypertension, and a low level of high-density lipoprotein cholesterol. Thus, hyperapoB shares characteristics with familial combined hyperlipidemia, LDL subclass pattern B, familial dyslipidemic hypertension, and syndrome X. Two metabolic defects have been described in patients with hyperapoB. In one, the hepatic synthesis of very-low-density lipoprotein is increased, resulting in the overproduction of small, dense LDL particles. In the other, there is a decreased clearance of postprandial triglycerides that is accompanied by an abnormal increase in free fatty acids.

The incorporation of free fatty acids into triglycerides in both adipocytes and cultured fibroblasts from hyperapoB subjects is about half that found in normal cells. Since these initial in vitro experiments were performed in the presence of lipoprotein-deficient serum, a partially purified serum protein was then isolated that stimulated the incorporation of free fatty acids into triglycerides in both normal adipocytes and fibroblasts.

Cianflone and coworkers identify this acylation stimulatory activity as residing in a small basic protein called acylation-stimulatory protein (ASP). ASP doubles the rate of incorporation of oleate into triglycerides in normal cultured fibroblasts, an effect that is reduced by about 50% in cells from patients with hyperapoB. Subsequently, the deficiency in acylation stimulatory activity with ASP has been found to be accompanied by a decrease in the high-affinity binding of 125I-ASP to fibroblasts from hyperapoB subjects.

We have isolated and partially characterized three basic proteins (BPs) from normal human serum that we call BP I, BP II, and BP III. Their apparent molecular weight and isoelectric point values are, respectively, BP I, 14 000 and 9.10; BP II, 27 500 and 8.48; and BP III, 55 000 and 8.73. BP I, BP II, and BP III differ in their amino acid compositions. BP I appears homologous to ASP. As judged by [14C]oleate incorporation into cellular lipids, BP I, BP II, and BP III each manifested acylation stimulatory activity in normal cultured fibroblasts. However, in hyperapoB cells, there was an approximately 50% defect in the stimulation of [14C]oleate incorporation by BP I into both cellular triglycerides and cholesteryl esters; in contrast, BP II stimulated (up to ninefold) the incorporation of [14C]oleate into cell cholesteryl esters in hyperapoB cells. No abnormality was noted with BP III in hyperapoB cells.

Here we have studied the effects of BP I, BP II, and BP III from normal human serum on the mass of triglycerides, cholesteryl esters, and phospholipids in normal and hyperapoB fibroblasts and have compared these effects with the rate of [14C]oleate incorporation into these cellular lipids. Since previous studies suggest that the effects of these proteins are mediated through...
a high-affinity process,\textsuperscript{17-22} we also examined whether inhibition or activation of protein kinase C may modulate the effects of these BPs on cell lipids.

\section*{Methods}

\subsection*{Patient Population}

Six hyperapoB probands (5 men and 1 woman) with at least one affected first-degree relative were studied.\textsuperscript{22} Five of the 6 had angiographically documented premature coronary atherosclerosis.\textsuperscript{4} The lipid, lipoprotein, and apolipoprotein B levels from these index cases and from 6 unrelated nonlipidemic control subjects have been published.\textsuperscript{22} Two children (Mi.H., normal, and Ma.H., hyperapoB) of one proband (C.H.) and the hyperapoB sister (T.L.) of another proband (B.O.) were also studied (Table 1).

\subsection*{Fibroblasts}

After the subjects gave informed consent, fibroblasts were grown from skin biopsies taken from the forearm as described.\textsuperscript{18} Cells were used between passages 5 to 15.

\subsection*{Protocol for Cell Experiments}

Fibroblasts (1×10\textsuperscript{5}) were grown in minimal essential medium containing 10% (vol/vol) fetal calf serum, 1% amino acids, 100 U penicillin/mL, and 100 mg streptomycin/mL for 6 days. The medium was then changed to a supplemented serum-free medium for 24 hours.\textsuperscript{22} At zero time, oleate/albumin (4:6:1; 10 mmol/L oleate) was added to medium without BPs (control cells) or to medium to which 6 \mu{g} of BP I (428.6 nmol/L), BP II (218.2 nmol/L), or BP III (109.1 nmol/L) had been added separately. The cells were then incubated for 6 hours.\textsuperscript{22} The concentrations of BPs used were those previously found to exert their maximal effects on lipid synthesis in fibroblasts.\textsuperscript{22} When the rate of incorporation of \textsuperscript{14}C\textsuperscript{oleate into lipid esters was determined, \textsuperscript{14}C\textsuperscript{oleate/albumin} (specific activity, 10 000 disintegrations per minute/nmol) was added to the medium. In one experiment, the rate of incorporation of \textsuperscript{3}H\textsuperscript{glycerol phosphate (specific activity, 9980 dpm/nmol) into phospholipids was also determined. The medium was then removed, the cells were washed, and the lips were extracted as described.\textsuperscript{22} An aliquot of the lipid extract was used to determine the amount of \textsuperscript{14}C\textsuperscript{oleate incorporated into the individual lipid esters by using the method of Goldstein et al.\textsuperscript{23} The mass of the individual lipid esters was also measured (see below). Sodium hydroxide was added to the cell residue and dried, and the cell protein content was determined by the method of Lowry et al.\textsuperscript{24} Duplicate dishes of cells were used for each condition. The values from the control cells grown in the absence of BP I, BP II, or BP III were subtracted from those grown in the presence of one of these proteins and were expressed as nanomoles \textsuperscript{14}C\textsuperscript{oleate per milligram cell protein per hour or as nanomoles cell lipid per milligram cell protein.

\subsection*{Mass Measurements of Lipids in Fibroblasts}

The mass of free and esterified cholesterol was determined by gas-liquid chromatography using a method modified after Ishikawa et al.\textsuperscript{25} Stigmasterol was used as an internal standard. For measurement of free cholesterol, the lipid extract was dried under a nitrogen stream, resolubilized in carbon disulfide, and injected into an HP-17 cross-linked 50% phenylmethylsilicone capillary column at 285\textdegree C using a helium flow of 30 mL/min. Total cholesterol was measured similarly after saponification. The mass of cholesterol that was esterified was calculated by subtracting the mass of free cholesterol from the mass of total cholesterol. The mass of triglycerides was determined enzymatically by using a commercially available kit (Seradyn Triglycerides Procedure, catalogue No. 47161). Lipid phosphorus was determined by using the method of Bartlett;\textsuperscript{26} the mass of total phospholipids was estimated by multiplying the phosphorus content by 25.

\subsection*{Second-Messenger Pathways}

For several experiments the cell protocol outlined above was followed, and the effects of BP I on \textsuperscript{14}C\textsuperscript{oleate incorporation into triglycerides and of BP II on \textsuperscript{3}H\textsuperscript{glycerol phosphate incorporation into cholesteryl esters in the presence and absence of I-(5-isouquinoline-sulfonyl)-2-methylpiperezine-dihydrochloride (H-7), an inhibitor of protein kinase C, were determined.\textsuperscript{22} We also studied the stimulation of protein kinase C by using C8, an analogue of diacylglycerol that activates protein kinase C.\textsuperscript{27} The effect of C8 on \textsuperscript{14}C\textsuperscript{oleate incorporation into triglycerides and cholesteryl esters in the presence and absence of BP I and BP II was studied.

\subsection*{Other Methods}

The amount of radiolabeled lipid incorporated into an individual lipid class was determined by liquid scintillation spectrometry. Tests of significance were performed by using Student's paired t test.

\subsection*{Results}

\subsection*{Mass of Cell Lipids in the Absence of BPs}

The mass of lipids in normal and hyperapoB fibroblasts grown in F-12 (control) medium without BPs was characterized (Table 2). There were no significant differences between the normal and hyperapoB cells.

\subsection*{Effects of BP I, BP II, and BP III on Cell Triglycerides}

In normal fibroblasts, BP I increased the mean mass of cell triglycerides about twofold over that in control medium, whereas there was significantly (P<.005) less stimulation in the production of triglycerides with BP I in the hyperapoB cells (Fig 1). The stimulation of \textsuperscript{14}C\textsuperscript{oleate into cell triglycerides seen in normal cells with BP I was also reduced about 50% in the hyperapoB

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Subject} & \textbf{Age, y} & \textbf{Sex} & \textbf{Total Cholesterol} & \textbf{Total Triglycerides} & \textbf{LDL Cholesterol} & \textbf{LDL-ApoB*} & \textbf{HDL Cholesterol} \\
\hline
\textbf{Normal} & & & & & & & \\
\hline
MiH & 29 & F & 4.47 & 0.56 & 2.27 & 0.0016 & 1.94 \\
\textbf{HyperapoB} & & & & & & & \\
MaH & 31 & M & 4.83 & 0.87 & 3.39 & 0.0023 & 1.11 \\
TL & 36 & F & 5.68 & 1.22 & 3.80 & 0.0028 & 1.65 \\
\hline
\end{tabular}
\caption{Plasma Lipid, Lipoprotein, and LDL-ApoB Levels in Three Relatives of HyperapoB Probands}
\end{table}

LDL indicates low-density lipoprotein; apo, apolipoprotein; hyperapoB, hyperapoB10lipoproteinemia; and HDL, high-density lipoprotein. Cholesterol and triglyceride levels are given in millimoles per liter.

*To convert to milligrams per deciliter, multiply by 55 000.
cells (P<.005) (Fig 1). BP II and BP III increased the mass of cell triglycerides in normal cells about 1.5-fold, but no significant abnormalities in triglyceride metabolism were found in the hyperapoB cells with either BP II or BP III (Fig 1).

Effects of BP I, BP II, and BP III on Cell Cholesteryl Esters

The increase in the mass of cell cholesteryl esters seen in the normal cells with BP I was significantly reduced (P<.005) in the hyperapoB cells (Fig 2). A similar reduction (P<.005) in the rate of incorporation of [14C]oleate into cell cholesteryl esters after treatment with BP I was also found in the hyperapoB compared with the normal cells (0.03 and 0.32 nmol [14C]oleate/mg cell protein per hour, respectively). In contrast, there was a significant sixfold increase in the stimulation of mass of cell cholesteryl esters in hyperapoB versus normal cells after treatment with BP II (P<.005). A similar abnormal increase in the stimulation of [14C]oleate incorporation into cholesteryl esters with BP II was also seen in the hyperapoB compared with the normal cells (0.26 and 0.04 nmol [14C]oleate/mg cell protein per hour, respectively). No abnormality in cholesteryl ester metabolism in hyperapoB cells was found with BP III (Fig 2).

The significant (P<.005) differences between normal and hyperapoB cells in cholesteryl ester mass after treatment with BP I (lower than normal) or BP II (higher than normal) were accompanied by parallel changes in the mass of total cholesterol, but this was not statistically significant (P=.09 for each) (Fig 2). No differences between normal and hyperapoB cells were seen in the pool of free cholesterol after treatment with BP I or BP II. Again, no abnormalities in hyperapoB cells were detected with BP III.

Effects of BP I, BP II, and BP III on Cell Phospholipids

After incubation with BP I there was about a twofold stimulation in the mass of total phospholipids over F-12 medium alone in the normal cells (Fig 3). This stimulation with BP I was reduced by about one third in the hyperapoB cells, but this was not statistically significant (P=.08). BP II and BP III stimulated the mass of total phospholipids much less than BP I; there were no significant differences in the effects of BP II or BP III on the mass of cell total phospholipids between the normal and hyperapoB cells (Fig 3). Similar trends for BP I were observed when the rates of [14C]oleate incorporation (Fig 3) or [3H]glycerol phosphate incorporation (data not shown) into cell phospholipids were studied.

Inhibition and Activation of Protein Kinase C

In normal fibroblasts, the stimulatory effect of BP I on [14C]oleate incorporation into triglycerides (in the absence of H-7) was decreased in a concentration-dependent fashion when H-7, an inhibitor of protein kinase C, was added (Fig 4, top). This effect of H-7 was more pronounced at a lower concentration in the hyperapoB cells (Fig 4, top). For BP II, we found in normal cells that H-7 decreased the incorporation of [14C]oleic acid into cell cholesteryl esters (Fig 4, bottom). The abnormal stimulatory effect of BP II on cholesteryl ester formation in hyperapoB cells was stimulated at a lower concentration of H-7 but was inhibited at higher concentrations of H-7 (Fig 4, bottom).

C:8, an analogue of diacylglycerol, was used to activate protein kinase C. In normal cells incubated in F-12 medium without BPs, C:8 stimulated [14C]oleate incorporation into triglycerides about fourfold over baseline; BP I nearly doubled this effect in both control and C:8-treated normal cells (Fig 5, top). In the hyperapoB cells, the addition of C:8 to F-12 medium alone stimulated the formation of triglycerides about fivefold; when BP I was added with C:8, little additional stimulatory effect with BP I occurred in the hyperapoB cells, whereas it increased 1.5-fold in the normal cells (Fig 5, top). In normal cells, BP II stimulated [14C]oleate incorporation into cholesteryl esters to a small degree; the addition of C:8 to either F-12 medium or BP II-containing medium did not stimulate cholesteryl ester formation (Fig 5, bottom). In hyperapoB cells, the abnormal stimulation of cholesteryl ester formation with BP II was similar in the presence or absence of C:8.

These data together suggest that protein kinase C activity per se is not deficient in the hyperapoB cells and that the abnormal cellular response of hyperapoB cells to BP I or BP II may occur at another, perhaps earlier, step in the pathway.

Discussion

The present studies confirm and extend our previous observations, namely that hyperapoB cells are deficient in their response to the acylation stimulatory activity of BP I (or ASP) and, in contrast, that hyperapoB cells are abnormally stimulated to form cholesteryl esters by BP II. Again, no abnormality was found with BP III.

In normal fibroblasts, BP I, BP II, and BP III increased the mass of cell triglycerides over that found in F-12 control medium (Table 2) about 2-, 1.5-, and 1.4-fold, respectively, increases that are similar to previous observations that used the rate of [14C]oleate incorporation as an index of the formation of triglycerides in fibroblasts. In hyperapoB fibroblasts, there was a 50% deficiency in the normal stimulation of the

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**TABLE 2. Mass of Lipids in Normal and HyperapoB Fibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides</th>
<th>Total Cholesterol</th>
<th>Free Cholesterol</th>
<th>Cholesteryl Esters</th>
<th>Total Phospholipids</th>
<th>Total Protein</th>
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<tbody>
<tr>
<td>Normal cells (n=7)</td>
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<td>HyperapoB cells (n=8)</td>
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HyperapoB indicates hyperapobetalipoproteinemia. Cells were grown in duplicate dishes for 6 hours in F-12 medium containing oleate/albumin (4.6:1; 10 mmol/L oleate). See "Methods" for details of experimental techniques. Measurements are given in nanomoles per milligram cell protein except for total protein, which is expressed as micrograms of protein per dish.

*Mean±1 SEM from three separate experiments.
mass of triglycerides with BP I, but no abnormal effect of BP II or BP III on triglyceride metabolism was observed in hyperapoB cells.

In normal fibroblasts, BP I also stimulated the formation of cell cholesteryl esters, an effect that was also diminished in hyperapoB cells (Fig 2). Compared with normal cells, the mass of cholesteryl esters was abnormally stimulated (over sixfold) by BP II in hyperapoB cells. Compared with the mass of triglycerides formed, however, the mass of cholesteryl esters perturbed is quantitatively less striking. The changes in the mass of cholesteryl esters were paralleled by similar changes in the mass of total cholesterol but not in the mass of free cholesterol, suggesting that the pool of cellular free cholesterol was not depleted to form cholesteryl esters. These data therefore raise the possibility of significant differences in the synthesis of cholesterol in normal and hyperapoB cells after treatment with BP I or BP II.
Kwiterovich et al  Effect of Basic Proteins on Lipid Mass in Fibroblasts

We also found evidence that BP I increased the mass of cell total phospholipids as well as triglycerides in normal cells. This suggests that BP I effects a general increase in the synthesis of cellular glycerol lipids. Similar observations using both \[^{14}C\]oleate and \[^{3}H\]glycerol phosphate support this general tenet. The failure of the notably lower stimulation of total phospholipid formation in hyperapoB cells with BP I to reach statistical significance may be due to the relatively small number of separate experiments performed or to the greater variability in measuring the total phospholipid content in these cells, compared, eg, with that for measuring the cell mass of triglycerides.\(^{20,28,29}\)

No significant differences were found in the mass of cellular lipids in the normal and hyperapoB cells in F-12 control medium without BPs. This suggests that the differences in the mass of lipids observed after the addition of BPs to the cells were not simply related to baseline differences in cellular lipid pools. Moreover, by subtracting the baseline values from those observed after addition of BPs, each cell line served as its own control.
The acylation stimulatory effect of the BPs might be due to a stimulation of lipid synthesis or to the inhibition of lipid hydrolysis. Using a pulse-chase protocol, we previously found no significant differences in the rate of disappearance of $[^{14}C]$oleate into lipid esters in normal fibroblasts. Teng and coworkers have presented evidence for a high-affinity binding site for $[^{14}C]$-ASP (or BP I) in normal cultured fibroblasts. Current studies are underway to further identify the nature of the high-affinity binding process and whether BP I, BP II, and BP III bind to the same, or different, cell surface receptors. It is also possible that the cell surface receptor for the BPs may be tissue specific, perhaps through the expression of different isoforms in various tissues, namely, adipocytes, hepatocytes, and macrophages.

The effects of BP I, BP II, and BP III on $[^{14}C]$-oleate incorporation into lipid esters in normal fibroblasts are both time and concentration dependent. The concentration curves are saturable and thus compatible with a high-affinity process. Cianflone and coworkers have presented evidence for a high-affinity binding site for $[^{14}C]$-ASP (or BP I) in normal cultured fibroblasts. Current studies are underway to further identify the nature of the high-affinity binding process and whether BP I, BP II, and BP III bind to the same, or different, cell surface receptors. It is also possible that the cell surface receptor for the BPs may be tissue specific, perhaps through the expression of different isoforms in various tissues, namely, adipocytes, hepatocytes, and macrophages.

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References


Fig 5. Bar graphs. Top, Cells were grown as described in "Methods" and were then incubated with C:8 (58.1 μmol/L) (cross-hatched bars) or without C:8 (black bars) in F-12 medium with (+) or without (−) basic protein (BP) I (428.6 nmol/L). The rate of incorporation of $[^{14}C]$-oleate into triglycerides was measured as described in "Methods" in two normal (MM, MiH; left) and two hyperapobetalipoproteinemia (hyperapoB) (GG, TL; right) cell lines. Data are an average from two separate experiments. Bottom, Cells were incubated with C:8 (58.1 μmol/L) (cross-hatched bars) or without C:8 (black bars) in F-12 medium with (+) or without (−) BP II (218.2 nmol/L). The rate of incorporation of $[^{14}C]$-oleate into cholesteryl esters was measured in two normal (MM, MiH; left) and two hyperapoB (GG, TC; right) cell lines. Data are an average from two separate experiments.


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