Differential Effect of Genistein on the Stimulation of Cholesterol Production by Basic Protein II in Normal and Hyperapob Fibroblasts

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Abstract—We studied further the basis for the abnormal effect of human serum basic protein II (BP II) on cholesterol production in hyperapobetalipoproteinemia (hyperapoB) fibroblasts and whether this effect involves protein tyrosine kinase phosphorylation (TKP). Genistein, a specific inhibitor of TKP was used as a probe. Compared with normal cells, BP II stimulated significantly the cellular mass of total cholesterol (6.4-fold), unesterified cholesterol (3.6-fold), and esterified cholesterol (6.7-fold) in hyperapoB fibroblasts. The addition of genistein to BP II in hyperapoB cells markedly inhibited these abnormal stimulatory effects of BP II on cell sterol mass. In normal cells, the addition of genistein to BP II produced an opposite effect: a marked stimulation in the mass of total (5.5-fold) and esterified (18.3-fold) cholesterol and a decrease in unesterified cholesterol (3.4-fold). These effects of genistein on the formation of cellular cholesterol by BP II were both time and concentration dependent. The inhibition of the stimulatory effect of BP II on cholesterol production by genistein in hyperapoB cells may be mediated through 3-hydroxy 3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of cholesterol biosynthesis, since the rate of incorporation of [14C]acetate, but not [3H]mevalonate, into unesterified cholesterol was decreased by genistein in the hyperapoB cells. When the mass of cell total cholesterol in the cells treated with BP II was subtracted from those treated with BP II plus genistein, a negative number was produced in each of the six hyperapoB cell lines, while each of the normal cell lines retained a positive number. The mean difference for the mass of total cholesterol between the hyperapoB and normal fibroblasts under these conditions was 128.2 nmol/mg cell protein, a difference that was separated by $3 SD$. This study supports further the tenet that there is a defect in the response of hyperapoB cells to BP II and that this defect results in an abnormality in cholesterol metabolism that appears mediated through a protein TKP-mediated process. (Arterioscler Thromb Vasc Biol. 1998;18:57-64.)

Key Words: tyrosine kinase phosphorylation • familial combined hyperlipidemia • atherosclerosis

Hyperapobetalipoproteinemia is a lipoprotein disorder that is prevalent in patients with premature CAD. HyperapoB is characterized by an increased number of small, dense LDL particles, a phenotype shared with familial combined hyperlipidemia, LDL subclass pattern B, familial dyslipidemic hypertension, and syndrome X. Two metabolic defects have been described in patients with hyperapoB. First, there is overproduction of apolipoprotein B and VLDL particles. Second, the clearance of postprandial triglyceride-rich particles is delayed, accompanied by an abnormal removal of FFA. The stimulation of the incorporation of FFA into triglycerides of adipocytes by a low-molecular-weight serum BP (called ASP) is deficient in hyperapoB cells. It has been postulated that this defect leads to the increase in postprandial FFA, which then fluxes back to the liver, leading to overproduction of apolipoprotein B and VLDL. We studied the role of certain human serum BPs in the pathogenesis of hyperapoB. We isolated and partially characterized three BPs from normal human serum, which we called BP I, BP II, and BP III based on their electrophoretic migration. Their $M_r$ and isoelectric points, respectively, were BP I, 14,000 and 9.10; BP II, 27,500 and 8.48; BP III, 55,000 and 8.73. The amino acid compositions of each were distinct from each other. BP I appears to be a different protein from ASP but has similar physiological effects. ASP has been reported to be the same protein as C3a des-Arg ($M_r$ 8000), a proteolytic cleavage product of C3, the third component of complement. In normal cultured fibroblasts, the major effect of BP I is a marked (twofold to threefold) stimulation of the rate of incorporation of [14C]oleate into triglyceride and of the mass of triglyceride. BP II and BP III have much less of an effect than BP I on triglyceride formation in normal cells. The biochemical effects of BP I, BP II, and BP III on triglyceride and cholesterol metabolism in hyperapoB fibroblasts are clearly different from each other. First, there is a 50% deficiency in the stimulation of triglyceride production by BP I in hyperapoB fibroblasts. No abnormality in triglyceride

Received February 28, 1997; revision accepted September 12, 1997.
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metabolism was seen with BP II or BP III in hyperapoB cells. Second, BP II (but not BP I or BP III) abnormally stimulated (about sixfold) the mass of cholesterol and cholesteryl esters in hyperapoB fibroblasts. If such an effect of BP II occurred in the liver of hyperapoB patients, it may further accentuate hepatic apolipoprotein B and VLDL production.

While there was no abnormality in triglyceride or cholesterol metabolism in hyperapoB fibroblasts with BP III, in cultured human monocyte-derived macrophages, BP III, but not BP I or BP II, significantly stimulated the formation of cell cholesteryl esters. This observation suggests the possibility that the effect of the serum BPs may be tissue specific and provides further evidence for structural and functional differences between BP I, BP II, and BP III. While abnormalities in the effects of BP I and BP II in hyperapoB may contribute to overproduction of VLDL, leading to overproduction of LDL and increased atherosclerosis, BP III may contribute to atherogenesis by stimulating cholesteryl ester deposition in macrophages.

In cultured fibroblasts, the effects of BP I and BP II are time and concentration dependent, suggesting that a high-affinity cell-surface mechanism is involved. In hyperapoB cells, the defect in the response to the stimulation of triglyceride formation by BP I is also time and concentration dependent and appears related to a deficiency in a high-affinity cell-surface mechanism. Conversely, the abnormality in the overresponse of hyperapoB cells to BP II by enhanced cholesterol formation is concentration dependent and saturable and appears due to an increased high-affinity interaction of BP II with the cell surface.

The biochemical effects of BP I are blocked by genistein, a specific inhibitor of protein TKP. For example, the stimulatory effect of BP I on the production of triglyceride was inhibited by 50% by genistein in normal cells to a level seen in hyperapoB cells. In contrast, no inhibition of this effect of BP I was seen with genistein in hyperapoB cells, suggesting that the defect in the response of hyperapoB cells to the acylation stimulatory activity of BP I may involve TKP.

Here we have studied further the basis for the abnormal effect of BP II on cholesterol metabolism in hyperapoB fibroblasts and whether this effect involves TKP. In the process, we made the serendipitous observation that when genistein is added to BP II, there is a striking but opposite effect on cholesterol production in normal and hyperapoB cells, leading to a marked difference in cell cholesterol mass between the two cell types.

**Methods**

**Human Materials**

Cultured fibroblasts from eight relatives from five kindreds with familial hyperapoB and six unrelated normal subjects were studied. The eight relatives have been previously characterized and include five hyperapoB probands, the hyperapoB sibling of one proband, and two children of another proband (one normal, one hyperapoB). Four of the five kindreds were ascertained through index cases with hyperapoB and one from the Johns Hopkins CAD Study. After informed consent was obtained, fibroblasts were grown from skin biopsies taken from the forearm as described. The research was approved by the Joint Committee on Clinical Investigation.

**Isolation and Characterization of BP II**

BP II was isolated from normal volunteers and characterized as previously described, with the following modifications. For the initial purification step to obtain a mixture of BPs, human serum (25 mL) was applied to a DEAE–Affi Gel Blue column (2.5 × 32 cm), and several unbound peaks containing a mixture of BPs eluted with 125 mL of 0.02 mol/L phosphate, pH 8.0. Fractions in peak I that did not react against an antibody to k light chain (a contaminating BP) were used to isolate BP II further by preparative SDS gel electrophoresis and electroelution. The final purified BP II did not react on Western blots to antibodies against C₃a des-Arg, k light chain, prealbumin, apolipoprotein A-I, sterol carrier protein-2, protein 422, or ASP.

**Protocol for Cell Experiments**

Fibroblasts were used between passages 5 and 10. Fibroblasts (10⁶) were seeded and grown for 6 days in minimum essential medium containing 10% (vol/vol) fetal calf serum, 1% amino acids, 100 U penicillin per milliliter, and 100 mg streptomycin per milliliter. The medium was then changed to a supplemented serum-free medium for 24 hours at 37°C. At zero time, oleate–albumin (4.6:1, 10 nmol/L oleate) was added to the medium without BP II (control cells) or to medium to which 6 μg/mL of BP II (218.2 nmol/mL) had been added, either in the presence or absence of the indicated concentration of genistein (0 to 370 nmol/mL medium; Calbiochem). The cells were then incubated for 6 hours at 37°C. The concentration of BP II was used that was previously found to exert its maximal effect on lipid synthesis in fibroblasts. The medium was then removed, the cells washed, and the lipids extracted as described. The mass of cellular cholesterol, unesterified cholesterol, and esterified cholesterol or the incorporation of radioactivity were measured (see below). Sodium hydroxide was added to the cell residue, dried, and the protein content determined by the method of Lowry et al. Duplicate dishes of cells were used for each experimental condition.

**Endogenous Synthesis of Cellular Cholesterol**

The incorporation of sodium [1-14C]acetate or RS-[5-3H(N)]mevalonolactone into cell unesterified cholesterol and esterified cholesterol was studied employing the method of Goldstein and Brown, modified as follows. Supplemented serum-free medium was added to confluent fibroblasts, and after 6 hours, 10 μL/mL medium of [1-14C]acetate (100 nmol/L, 0.1 μCi/μL) and 10 μL/mL medium of [3H]mevalonolactone (33 nmol/L, 0.1 μCi/μL) were added to the medium and the incubation continued for another 18 hours. Oleate–albumin (4.6:1, 10 nmol/L oleate) was then added to the medium without BP II (control cells) or to a medium to which 6 μg of BP II (218.2 nmol/mL) was added, with or without genistein, and the incubation continued for another 6 hours. The medium was removed, the cells washed, and the lipids extracted as described. The lipid extract was dried, taken up in hexane, and the radioactivity determined by scintillation counting. The data are expressed as nanomoles [1-14C]acetate or [3H]mevalonolactone incorporated per milligram cell protein.
Mass Measurements of Cholesterol in Fibroblasts

The mass of total cholesterol, unesterified cholesterol, and esterified cholesterol was determined by GLC as described before. Total cholesterol was measured after saponification. The mass of cholesterol that was esterified was calculated by subtracting the mass of unesterified cholesterol from the mass of total cholesterol. The data are expressed as nanomoles cholesterol per milligram cell protein.

GLC/Mass Spectrometry

The identity and purity of cell cholesterol was determined by combined GLC/mass spectrometry of the trimethylsilyl ether esters, using a Hewlett Packard 5970A mass selective detector as described previously.

Statistical Analysis

Tests of significance within a group were performed by using a Student’s paired t test, and tests between the normal and hyperapoB cell lines were performed using the two-sample t test.

Results

Dose Response of the Effect of Genistein on the Formation of Cellular Cholesterol by BP II

The effect of increasing concentrations of genistein on the mass of total cholesterol, unesterified cholesterol, and esterified cholesterol in normal and hyperapoB cells grown in medium containing F-12 alone, F-12 plus BP II (uncorrected data), and BP II minus F-12 data (corrected data) was determined (Fig 1). Without any genistein in the medium, BP II stimulated the mass of total cholesterol, unesterified cholesterol, and esterified cholesterol in the hyperapoB cells, whereas little stimulation was observed in the normal cells (Fig 1). In F-12 medium alone, increasing concentrations of genistein decreased the cellular content of total cholesterol, unesterified cholesterol, and esterified cholesterol in both the normal and hyperapoB cells; these changes in the cell sterol content with genistein in the normal and hyperapoB cells were parallel with each other (Fig 1).

In medium containing F-12 plus BP II, the abnormal stimulatory effect of BP II in hyperapoB cells on sterol mass was inhibited by genistein in a dose-dependent fashion, reaching a nadir at a concentration of 92.5 nmol of genistein per milliliter of medium (Fig 1; uncorrected data). In distinct contrast, in the presence of genistein, BP II produced in normal cells a marked stimulation in total cholesterol mass, which reached a peak at 92.5 nmol of genistein per milliliter of medium (Fig 1). A similar pattern of diametrically opposite effects of genistein on the cell sterol mass of normal and hyperapoB cells in the presence of BP II was observed when the cell sterol data in medium containing F-12 alone were subtracted from that containing F-12 plus BP II (corrected data). The effects of genistein on cell sterol mass in the presence of BP II in normal and hyperapoB cells produced a dose-response curve that had a crossover or X configuration (Fig 1). In the normal cells, the paradoxical stimulation of total cholesterol in the presence of BP II plus genistein resided in the esterified cholesterol fraction (Fig 1), whereas in the hyperapoB cells, the abnormal stimulatory effect of BP II on both unesterified and esterified cholesterol in hyperapoB cells was inhibited by genistein (Fig 1).

Time Course of the Effect of Genistein on the Formation of Cellular Cholesterol by BP II

The abnormal stimulation of the cell mass of total cholesterol, unesterified cholesterol, and esterified cholesterol with BP II in hyperapoB cells was time dependent and appeared to reach a maximum by 6 hours (Fig 2). Addition of genistein (92.5 nmol/mL) and BP II to the hyperapoB cells obliterated this stimulation of BP II within 1 hour and maintained its inhibition (Fig 2). In normal cells, BP II alone had little effect on the mass of cell cholesterol. However, when genistein was added with BP II to the normal cells, there was a time-dependent stimulation in the mass of total and esterified cholesterol, but not of unesterified cholesterol, an effect that appeared to reach a maximum at 6 hours (Fig 2).

Effect of BP II With and Without Genistein on Cholesterol Mass in Normal and HyperapoB Fibroblasts

BP II alone significantly changed the cellular sterol mass and stimulated total cholesterol 6.4-fold (P<.0001), unesterified cholesterol 3.6-fold (P=.0006), and esterified cholesterol 6.7-fold (P=.0002) greater in the hyperapoB cells than in the normal fibroblasts (Fig 3). In hyperapoB cells, the addition of genistein to BP II markedly inhibited the abnormal stimulation by BP II of the mass of total cholesterol by 96% (P<.0001), of unesterified cholesterol by 78% (P=.0003), and of esterified cholesterol by 100% (P=.0017) (Fig 3). In normal cells, the addition of genistein to BP II produced an opposite cellular effect; a marked stimulation (5.5-fold) in the mass of total cholesterol in the hyperapoB cells, whereas little stimulation was observed in the normal cells (Fig 1).
cholesterol was observed ($P=.0001$) (Fig 3). Most of this increase of cholesterol in normal cells occurred in the esterified cholesterol fraction (18.3-fold) ($P=.0001$), while a significant decrease (3.4-fold) occurred in the stimulation of the mass of unesterified cholesterol ($P=.01$) (Fig 3).

Because of this marked increase in the stimulation of the cell sterol content in normal cells, the identity and purity of the cell sterols in two of the normal cell lines treated with BP II plus genistein were studied by combined GLC/mass spectrometry (see “Methods”). The single peak on GLC was found to be entirely cholesterol, as judged by its mass spectrum (mass ion of trimethylsilyl cholesterol, 458).

**Effect of BP II With and Without Genistein on Cholesterol Synthesis in Normal and HyperapoB Fibroblasts**

HMG CoA reductase is the rate-controlling step in cholesterol synthesis in human fibroblasts. Inhibition of this enzyme or an effect on its regulation that reduces its activity or quantity should result in decreased synthesis of cholesterol from acetate but not from mevalonate.

$[^{14}C]Acetate$ Incorporation Into Cell Unesterified and Esterified Cholesterol in F-12 Medium With and Without BP II

After 24 hours in F-12 lipid-deficient medium, there was no significant difference in the rate of $[^{14}C]acetate$ incorporation into either unesterified or esterified cholesterol between the normal and hyperapoB cells (data not shown). In the normal cells, addition of BP II to the F-12 medium did not significantly increase $[^{14}C]acetate$ incorporation (mean [SE] in nanomoles per milligram cell protein per hour), into unesterified cholesterol, 9.7 (1.9) versus 10.6 (1.3); $P=.75$. In contrast, BP II increased $[^{14}C]acetate$ incorporation into unesterified cholesterol in the hyperapoB fibroblasts from 7.8 (1.4) to 16.2 (1.4); $P=.0003$. Addition of BP II to F-12 medium produced a threefold increase in the rate of incorporation of $[^{14}C]acetate$ into esterified cholesterol (0.30 to 0.91; $P=.01$) in normal cells and a 12-fold increase (0.43 to 5.26; $P=.0025$) in the hyperapoB cells.

The data were corrected for the control condition to compare these effects of BP II in normal and hyperapoB fibroblasts (Fig 4). BP II stimulated $[^{14}C]acetate$ incorporation into unesterified cholesterol to a significantly greater extent in the hyperapoB cells than in the normal cells ($P=.0002$) (Fig 4). The addition of genistein to BP II–containing medium completely inhibited the abnormal stimulation of $[^{14}C]acetate$ incorporation into unesterified cholesterol in the hyperapoB cells ($P=.0001$) (Fig 4), whereas in normal cells, genistein plus BP II increased such incorporation, but to a small extent ($P=.19$) (Fig 4). Compared with normal cells, BP II stimulated $[^{14}C]acetate$ incorporation into esterified cholesterol in the hyperapoB cells eightfold ($P=.001$) (Fig 4). The addition of genistein to BP II–containing medium inhibited this abnormal stimulation of esterified cholesterol in hyperapoB fibroblasts by 66% ($P=.0003$) (Fig 4), whereas in normal cells, there was a small stimulation ($P=.11$) (Fig 4).

$[^{3}H]Mevalonolactone$ Incorporation Into Cell Unesterified and Esterified Cholesterol in F-12 Medium With and Without BP II

After 24 hours in lipid-free medium, the rate of incorporation of $[^{3}H]mevalonolactone$ (mean [SE] in nanomoles per milligram cell protein per hour), into unesterified cholesterol was
either the normal (5.25) or the hyperapoB cells (5.47) was then added to the medium without BP II (control cells) or with 6 μg/mL BP II (218.2 nmol/L), either in the absence or presence of 92.5 nmol/mL genistein. Cells were then incubated for 6 hours, the medium was removed, the cells were washed, and the lipids extracted. One aliquot of the lipid extract was used to determine the mass of total cholesterol, unesterified cholesterol, and esterified cholesterol (see Fig 3), while another aliquot was used to determine the incorporation of [14C]acetate (left) and [3H]mevalonolactone (right) into unesterified cholesterol (top) and esterified cholesterol (bottom), isolated by thin-layer chromatography (see “Methods”). The protein content of the cell residue was determined. Duplicate dishes of cells were used for each experimental condition. The data for [14C]acetate represent the average of two separate experiments, while the data for [3H]mevalonolactone represent one experiment. The incorporation of [14C]acetate and [3H]mevalonolactone into cell sterols from the control cells grown in the absence of BP II (with and without genistein) were subtracted from those grown in the presence of BP II (with or without genistein). Standard error bars are shown. Significant differences between the means are found in the text.

Figure 4. Effect of genistein on the stimulation of the incorporation of [14C]acetate and [3H]mevalonolactone into cholesterol in normal and hyperapoB fibroblasts treated with BP II. Confluent fibroblasts from four normal and four hyperapoB subjects were switched to a supplemented serum-free medium for 6 hours, at which point 10 μg/mL [14C]acetate (100 nmol/L, 0.1 μCi/μL) and 10 μg/mL [3H]mevalonolactone (33 nmol/L, 0.1 μCi/μL) were added, and the incubation in serum-free medium was continued for an additional 18 hours. Oleate-albumin (4.6:1, 10 nmol/L oleate) was then added to the medium without BP II (control cells) or with 6 μg/mL BP II (218.2 nmol/L), either in the absence or presence of 92.5 nmol/mL genistein. Cells were then incubated for 6 hours, the medium was removed, the cells were washed, and the lipids extracted. One aliquot of the lipid extract was used to determine the mass of total cholesterol, unesterified cholesterol, and esterified cholesterol (see Fig 3), while another aliquot was used to determine the incorporation of [14C]acetate (left) and [3H]mevalonolactone (right) into unesterified cholesterol (top) and esterified cholesterol (bottom), isolated by thin-layer chromatography (see “Methods”). The protein content of the cell residue was determined. Duplicate dishes of cells were used for each experimental condition. The data for [14C]acetate represent the average of two separate experiments, while the data for [3H]mevalonolactone represent one experiment. The incorporation of [14C]acetate and [3H]mevalonolactone into cell sterols from the control cells grown in the absence of BP II (with and without genistein) were subtracted from those grown in the presence of BP II (with or without genistein). Standard error bars are shown. Significant differences between the means are found in the text.

Differential Effect of Genistein Plus BP II in Normal and HyperapoB Fibroblasts

The individual data points for total cell cholesterol from six normal and six hyperapoB fibroblasts under the different conditions of cell culture before and after genistein treatment are schematically depicted in Fig 5, and the means and SDs are summarized in the Table. In F-12 medium alone, there was no difference between the normal and hyperapoB cells in the mass of total cholesterol (Fig 5 and Table). When genistein alone was added to F-12 medium, the mass of total cholesterol decreased significantly in both normal and hyperapoB cells; while the group mean was significantly lower in the hyperapoB cells, there was considerable overlap between the two cell types (Fig 5 and Table). When BP II alone was added to the F-12 medium, the total cholesterol mass increased significantly in the hyperapoB cells, and there was no overlap between the two cell types (Fig 5 and Table). However,
cells was governed by a TKP-mediated process that was stimulatory effect of BP I on triglyceride formation in normal BP II in hyperapoB cells. Previously we found that the completely the abnormal stimulation of cholesterol production by ATP in the tyrosine kinase reaction,19,20 inhibited com-

This study supports further the tenet that the cellular effects of the human serum BPs are mediated through a protein TKP-
defects in both cholesterol and triglyceride metabolism in hyperapoB cells that are related to the effects of BP II and BP I, both of which are mediated through TKP.

The effect of genistein on the biochemical actions of BP II was both time and concentration dependent, suggesting a high-affinity mechanism of genistein on the effects of BP II.24 The precise point of action of genistein on the effects of BP II remains to be determined. A generalized inhibition of several tyrosine kinases by genistein was taken into account, in part, by also determining the effect of genistein alone on cellular sterol in the control condition. The patterns of the effects of genistein on the actions of BP II in normal and hyperapoB cells were similar when using uncorrected sterol data or data corrected for the effects of genistein in F-12 medium alone. The marked stimulation of the mass of total cholesterol and esterified cholesterol when genistein was added to BP II in the normal cells, the fact that the effect of genistein was dose dependent and occurred at lower concentrations (23.1 nmol/mL medium), and the failure of genistein to decrease cellular protein argue against general cytotoxicity as the explanation for genistein’s inhibition of the stimulatory effect of BP II in hyperapoB cells. Genistein may have cellular effects other than inhibition of TKP. In that regard, we have direct immunochemical evidence in hyperapoB cells that BP II increases TKP of hyperapoB fibroblast proteins25 and that genistein prevents such stimulation of TKP by BP II (see also below).

Discussion
This study supports further the tenet that the cellular effects of the human serum BPs are mediated through a protein TKP-dependent process. Genistein, a specific competitive inhibitor of ATP in the tyrosine kinase reaction, inhibited completely the abnormal stimulation of cholesterol production by BP II in hyperapoB cells. Previously we found that the stimulatory effect of BP I on triglyceride formation in normal cells was governed by a TKP-mediated process that was deficient in hyperapoB cells.11 Thus, there appear to be cellular errors in the table.
II in hyperapoB cells. This abnormal cellular effect of BP II was inhibited by genistein, implicating TKP in its pathogenesis. There appears to be a defect in the hyperapoB cells that causes them to overrespond to BP II; in contrast, there is a significant underresponse of hyperapoB cells to BP I.\textsuperscript{8–11} While both cellular abnormalities apparently involve a second-messenger pathway inhibited by genistein, the data do not allow us to differentiate whether the effects of genistein involve a transmembrane tyrosine kinase receptor, a membrane-associated tyrosine kinase molecule,\textsuperscript{26,27} or a nonmembrane postreceptor molecule\textsuperscript{28} (see also below). Our recent data\textsuperscript{25} indicate that there is considerably less immunoreactivity of phosphotyrosine membrane proteins to a monoclonal antiphosphotyrosine antibody in hyperapoB cells grown in F-12 medium alone than in normal fibroblasts. The immunoreactivity of several of these proteins is increased by BP II in hyperapoB cells, but not in normal fibroblasts. This effect of BP II is inhibited by genistein in hyperapoB cells. HyperapoB cells also appear to manifest enhanced high-affinity binding of BP II but reduced binding of BP I compared with normal cells.\textsuperscript{12} One possible explanation is that there is a defect in a transmembrane tyrosine kinase receptor in hyperapoB cells, leading to abnormalities in TKP with BP II and BP I.\textsuperscript{1,8–12}

We had previously found that an inhibitor of protein kinase C decreased the abnormal stimulation of cell esterified cholesterol by BP II in hyperapoB fibroblasts.\textsuperscript{10} Nonreceptor tyrosine protein kinases, such as phospholipase C \(\gamma\), that contain \(SH_2\) and \(SH_3\) domains may become physically associated with, and phosphorylated by, an activated transmembrane receptor protein tyrosine kinase. Following TKP, phospholipase C \(\gamma\) cleaves phosphatidylinositol 4,5-diphosphate into second messengers diacylglycerol and inositol triphosphate, which then stimulate protein kinase C and mobilize Ca\textsuperscript{2+}, respectively.\textsuperscript{27} The molecular defect in hyperapoB cells may reside either in a transmembrane receptor protein tyrosine kinase or in a prototype cytoplasmic signaling protein such as phospholipase C \(\gamma\). However, as with insulin (or other hormones or cytokines), one cannot conclude that all the cellular responses to BP II depend on receptor or cell surface–associated tyrosine kinase activity alone. For example, genistein itself has been shown in rat adipocytes to inhibit differentially the postreceptor effects of insulin, but without inhibiting the insulin receptor tyrosine kinase.\textsuperscript{24}

When BP II stimulated abnormally the formation of cell esterified cholesterol in hyperapoB fibroblasts, the pool of cell unesterified cholesterol was not depleted,\textsuperscript{26} suggesting that there may be an effect of BP II on cholesterol biosynthesis in hyperapoB cells treated with BP II and prompting us to examine cholesterol production. Our data here indicate that BP II abnormally stimulates the production of cholesterol in hyperapoB fibroblasts. In hyperapoB cells, BP II might increase the transcription of the \(HMG\text{CoA}\) reductase gene, stabilize its steady state of mRNA level, or enhance its translation, and further experiments are necessary to elucidate these possible mechanisms. The inhibition of this effect of BP II by genistein may be mediated through HMG \(\text{CoA}\) reductase, the rate-limiting enzyme of cholesterol biosynthesis, because the incorporation of \(^{14}\text{C}\text{acetate}\), but not \(^{1}\text{H}\text{mevalonolactone}\), into unesterified cholesterol was decreased by genistein in hyperapoB cells. Sato and coworkers\textsuperscript{28} showed that phosphorylation of a serine in \(HMG\text{CoA}\) reductase decreases its catalytic activity. Thus, any inhibition of phosphorylation of \(HMG\text{CoA}\) reductase by treatment with genistein would need to be indirect, ie, by inhibiting the activation of the protein responsible for serine phosphorylation. Furthermore, such an effect would be expected to increase, not decrease, cholesterol biosynthesis. The decrease in cholesterol production by genistein in hyperapoB cells is therefore unlikely to be due to inhibition of phosphorylation of \(HMG\text{CoA}\) reductase.

It is not known under the various conditions of cell culture employed in this study whether BP II or genistein affected the transport of cholesterol from the cells into the medium. The medium, however, was not saved in these experiments, and no sterol data could be obtained for the medium. The cells were grown in lipid-free medium for 24 hours before the incubation with BP II with or without genistein and continued in cholesterol-free medium for 6 hours. However, the cells were always incubated in presence of albumin–FFA complex, which may have served as a possible acceptor of cellular lipids. It is also not known whether BP II or genistein may effect the secretion of lipid-binding proteins by cells into the medium.

A major, albeit unexpected, observation of this study was the marked stimulation of the mass of cholesterol in normal cells that occurred when genistein was present in the medium along with BP II. Without BP II, genistein alone decreased cell cholesterol mass. BP II did not simply reverse the effect of genistein alone, because under those circumstances, one would expect the mass of cell cholesterol to return to the level seen in the presence of F-12 medium alone (Fig 5). This combined effect of BP II and genistein on sterol metabolism in normal fibroblasts was accompanied by a small increase in cholesterol production. However, the increase in the mass of esterified cholesterol appeared to occur, in part, at the expense of the stimulation of the mass of unesterified cholesterol, suggesting an increased conversion of unesterified cholesterol to esterified cholesterol, which may occur by either an increased activity of acyl cholesterol acyltransferase, or a decreased activity of cholesteryl ester hydrolase. However, not all of the increase in cell esterified cholesterol appeared to be accounted for by these proposed mechanisms, and further experiments will be necessary to determine whether there was an increased flux of substrate (ie, acetyl-CoA) that may have diluted the \(^{14}\text{C}\text{acetate}\) pool, leading to an underestimation of the rate of cholesterol production.

The marked difference between normal and hyperapoB fibroblasts in their cholesterol content when they were treated with BP II in the absence or presence of genistein provided a separation between the hyperapoB and normal cell types that was at least as large as that seen for LDL cholesterol between normal subjects and those patients with the autosomal-dominant disorder familial hypercholesterolemia.\textsuperscript{29} A similar degree of separation between these normal and hyperapoB cells was also found for \(\text{TKP}\) of fibroblast proteins.\textsuperscript{30} The simplest explanation for these data is that the abnormal cellular phenotype described here reflects the expression of the primary genetic defect. While the primary defect has not been established, it probably involves TKP (as discussed above). Furthermore, families with hyperapoB and familial combined hyper-
lipidemia are undoubtedly genetically heterogeneous, and these data are confined to six unrelated families ascertained through probands with premature CAD and hyperapoB. We are currently studying what proportion of probands with premature CAD and hyperapoB manifests this abnormal cellular phenotype and whether the defect cosegregates with hyperapoB in these families. Specificity and sensitivity are also issues to be resolved.

Our data to date indicate a cellular defect in hyperapoB fibroblasts in response to serum BPs isolated from normal control subjects. We have not yet purified BPs from hyperapoB patients. It is possible that some hyperapoB patients may have defects in the human serum BPs themselves that might lead to abnormal cellular responses. We are currently developing monoclonal and polyclonal antibodies to the human serum BPs to ELISA assays to quantitate the serum levels of human BPs in normal patients and those hyperapoB patients with the cellular defects. Once these two groups are characterized, well-defined kindreds with familial hyperapoB will be screened to identify probands with either qualitatively different reactions of their human serum BPs to the immunologic reagents or quantitative patterns that indicate that they may differ in some way. Such families may be ideal candidates to purify the BPs from hyperapoB subjects.

In conclusion, these observations provide further evidence that there is a defect in TKP in hyperapoB cells and that such a defect involves cholesterol as well as triglyceride production. Current studies are in progress to determine the molecular basis for these observations.

Acknowledgments

This work was supported by the following grants from the National Institutes of Health: 1 P50 HL47212 (Specialized Center of Research In Arteriosclerosis), HL 31497 to 05, 1 R01 HD32193 to 03, General Clinical Research Center Program, RR-52, RR-35, and CLINFLO. We thank Pauline Gugliotta and Donna Virgil for their help in preparing this manuscript. We also thank Dr Pascal Goldschmidt for stimulating conversations on the possible role of protein tyrosine kinase phosphorylation in the pathogenesis of hyperapoB and Dr Paul Bachorik for reviewing the manuscript.

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Arterioscler Thromb Vasc Biol. 1998;18:57-64
doi: 10.1161/01.ATV.18.1.57

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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