Signal transduction pathway of Acylation Stimulating Protein: involvement of protein kinase C

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Abstract Acylation Stimulating Protein (ASP) was recently purified to homogeneity from human plasma and shown to be identical to C3adesArg. ASP stimulates triglyceride synthesis in human skin fibroblasts and primary human adipocytes. In vitro differentiation of human preadipocytes to mature fat cells results in increased expression and accumulation of ASP in the medium. These differentiated human adipocytes are also much more responsive to ASP than preadipocytes. The object of this study was to investigate the signal transduction pathway by which ASP causes triglyceride synthesis (TGS) to increase in human cultured fibroblasts and adipocytes. No evidence was found for a protein kinase A-mediated response. ASP action was consistent with a protein kinase C (PKC)-mediated pathway in that: *1)* the effect of ASP on TGS was mimicked by 1–10 nM phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC; (202% ASP vs. 178% PMA stimulation); *2)* the effect of PMA and ASP were non-additive with respect to TGS; *3)* staurosporine (50 nM) and GF109203X (bisindolylmaleimide) at 1 μM, both competitive inhibitors of the ATP-binding site on PKC, inhibited both ASP and PMA stimulation of TGS (–59% and –65% for ASP and –84% and –99% for PMA, respectively); *4)* Calphostin C (0.8 μM) which interacts with the regulatory domain of PKC also inhibited the ASP- and PMA-mediated stimulation of PKC (–76% ± 11% inhibition for ASP and –99% ± 20% inhibition for PMA), although in all cases the inhibition of PMA-stimulated triglyceride synthesis was greater; *5)* ASP caused a time-dependent increase in intracellular diacylglycerol accumulation; and finally *6)* stimulation by ASP caused an increase in PKC activity and a time-dependent translocation of PKC (maximal effect at 30 min) from the soluble intracellular compartment to a membrane-bound fraction (basal activity 22% in the membrane-bound fraction, ASP 54%, $P < 0.05$ and PMA 69% $P < 0.0025$). Taken together, the data are consistent with the conclusion that ASP acts to stimulate triglyceride synthesis via activation of the protein kinase C pathway.—**Baldo, A., A. D. Sniderman, S. St. Luce, X-J. Zhang, and K. Cianflone.** Signal transduction pathway of Acylation Stimulating Protein: involvement of protein kinase C. *J. Lipid Res.* 1995. **36:** 1415–1426.

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Until recently, there has been little evidence that the rate of adipocyte triglyceride synthesis is physiologically regulated. This concept, however, was challenged by the isolation of a human plasma protein that causes triglyceride synthesis in cultured human skin fibroblasts (1) and adipocytes (2) to increase markedly. Based on this effect, this protein was named Acylation Stimulating Protein (ASP). ASP levels increase in human plasma postprandially and the degree to which they increase correlates directly with the rate at which triglycerides are cleared from plasma (3). We have recently shown that ASP is identical to C3adesArg, a biologic fragment of human complement factor C3 generated through the alternate complement pathway (1). Moreover, it is now evident that both murine and human adipocytes can generate ASP (2, 4). Additionally, differentiated adipocytes are more responsive to ASP than preadipocytes, thus establishing the presence of an adipsin/ASP pathway in human adipose tissue.

Studies using cultured human skin fibroblasts and human adipose tissue microsomes have shown that ASP increases the rate of triglyceride synthesis through a coordinate effect on two separate aspects of the triglyceride synthetic pathway (5, 6). First, ASP increases the rate of entry of glucose into the cells by promoting the translocation of glucose transporters from an intracellular pool to the plasma membrane (5). This results in an increase in the intracellular availability of glucose

Abbreviations: ASP, Acylation Stimulating Protein; ACN, acetonitrile; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; PMA, 4 β-phorbol 12-myristate 13-acetate; FCS, fetal calf serum; TGS, triglyceride synthesis; PKC, protein kinase C; NS, not significant; hyperapoB, apoB hyperlipoproteinemia; DAG, diacylglycerol; MBP, myelin basic protein.

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which acts as the precursor of the backbone of the triglyceride molecule, glycerol-3-phosphate. Second, ASP enhances the overall rate of triglyceride synthesis by increasing the rate of esterification of fatty acids to the glycerol-3-phosphate backbone (5). ASP does so by increasing the activity of diacylglycerol acyltransferase (DGAT), the enzyme catalyzing the final and committed step in the synthesis of triglyceride (6). Kinetic analysis indicates that both the increase in glucose transport and the increase in triglyceride synthesis are mediated through changes in V_{max} and not K_m, suggesting, therefore, that ASP affects overall activity, not substrate binding (5, 6).

Understanding the molecular mechanism of action of ASP is, therefore, of considerable importance. Based on our data on cell specific interaction (7) and the subsequent coordinate response in triglyceride synthetic and glucose transporter activity (5) we propose the following: *i)* ASP acts through interaction with a postulated cell surface receptor; *ii)* interaction with the receptor mediates generation of a second messenger which activates one of the signal transduction pathways. There are a number of previous reports demonstrating that phorbol esters stimulate triglyceride synthesis via a protein kinase C pathway in rat adipocytes (8, 9) cultured fibroblasts (10), and other cells (11) and glucose transport in isolated rat adipocytes and cultured myocytes (12, 13). In addition, it has been reported that cholera toxin also stimulates glucose transport in both fibroblasts and adipocytes through a non-cAMP protein kinase mechanism (14, 15). Accordingly, this report presents the results of experiments designed to examine the potential involvement of second messenger generation in the signal transduction pathway of ASP.

METHODS

Purification of Acylation Stimulating Protein

General chemicals and solvents were from Fisher Scientific (Nepean, Canada). Acylation Stimulating Protein was isolated from frozen plasma. Frozen plasma was thawed overnight at 4°C, warmed quickly with stirring to 37°C, and spiked with inulin (0.5% w/v final concentration) and with 1 M $MgCl_2$ solution (2 mM final concentration). The spiked plasma was stirred for 1 h at 37°C to enzymatically maximize in vitro generation of ASP from endogenous complement C3 (16) and increase the final yield of ASP. The plasma was transferred to an ice bath and globular proteins were precipitated by addition of concentrated HCl (1 M final concentration). The plasma was centrifuged at 4000 *g* for 30 min and the supernatant was neutralized to pH 7.4 by the addition of 10 N NaOH. A C-18 Sep-Pak column (Waters-Millipore, Mississauga, Ontario), 40 g/2 L of plasma was pre-equilibrated with 5 volumes of 80% ACN–0.1% TFA (acetonitrile–0.1% trifluoroacetic acid v/v) followed by 10 volumes of 0.1% TFA prior to use. The plasma supernatant was then applied to the column and the column was washed with 10 volumes (until absorbance at 280 nm returned to baseline) of 20% ACN–0.1% TFA. A single protein peak was eluted from the column with 10 volumes of 80% ACN–0.1% TFA and collected as 10-mL fractions (absorbance at 280 nm). The elution peak (average 60 mL) was pooled and loaded on an S-Sepharose column (Pharmacia LKB Biotechnology Products, Baie d'Urfe, Quebec, Canada) 20 mL/bed volume/L starting plasma that had been previously pre-equilibrated with 5 volumes of buffer A (10 mM Tris, 10 mM NaCl, pH:7.1). After loading the sample, the column was washed with 5 volumes of buffer A (until absorbance at 280 nm returned to baseline) and eluted with 10 volumes of 1 M NaCl in buffer A. The elution peak (average 100 mL) was pooled and loaded on a semi-preparative Vydac Protein C4 column (1.0 × 25 cm, Separations Group, Hesperia, CA) pre-equilibrated with 0.1% TFA (5 runs/2 L starting plasma). Loading and elution was carried out at 2.0 mL/min with absorbance monitoring at 280 nm. After a 20-min wash of the column with 24% ACN/0.1% TFA, proteins were eluted from the column and fractionated (3 mL/tube) with a linear gradient from 24% ACN/0.1% TFA to 80% ACN/0.1% TFA in 45 min. The ability of column fractions to stimulate triglyceride synthesis was tested as previously described (1). Tubes from the peak containing triglyceride synthesis-stimulating activity from sequential runs were pooled.

Following this protocol, ASP typically eluted from the Vydac C4 column at 50% ACN. To the active pool was added 2 mL of a sterile solution of 10 mg/mL sterile essentially fatty acid-free BSA in water (Sigma, St. Louis, MO). The pool was aliquoted into 20 siliconized glass vials (Sigmacote, Sigma, St. Louis, MO; see supplier's instructions) and lyophilized in a centrifuge evaporator. Lyophilized aliquots were reconstituted in 1 mL sterile phosphate-buffered saline (PBS) with gentle vortexing and stored at –80°C. Average ASP yield for each purification step is shown in **Table 1.** It should be noted that ASP should be stored at –80°C in siliconized glass vials to reduce aggregation, sticking to surfaces, and other nonspecific losses. Average yield from 1 L plasma was 19 ± 3.3 mg as measured by commercial radioimmunoassay for C3adesArg (Amersham, Oakville, Canada).

Culture of human skin fibroblasts

Fibroblasts were obtained from forearm skin biopsies of normolipidemic subjects. Primary cultures were established from explants and maintained in Minimum

TABLE 1. ASP purification from plasma

Purification Step	Protein[a]	ASP[b]	ASP Recovery	% ASP/ Total Protein	-Fold Purification
	mg	*mg*	%	%	
Plasma (2 L) post Mg^{2+} and inulin	95,167 ± 4,204	258.8 ± 39.8	100	0.3	1.3
Acid supernatant	9,249 ± 1,199	215.8 ± 32.4	87 ± 7	2.3	11.0
Post Sep-Pak	111 ± 23	65.0 ± 18.5	24 ± 7	58.5	279.0
Post S-Sepharose	104 ± 16	51.6 ± 9.1	20 ± 5	49.7	236
HPLC C-4	33 ± 6	38.1 ± 6.2	14 ± 2	100.0	476

[a]Protein determined by Bio-Rad dye-binding colorimetric commercial assay.
[b]ASP (C3adesArg) determined by commercial RIA assay. Note that ASP as determined by RIA yields values slightly greater than the dye-binding assay.

Essential Medium (MEM) with 10% fetal calf serum. MEM and Dulbecco's minimum essential medium/F12 (D-MEM/F12), Dulbecco's-phosphate buffered saline (D-PBS) and all other tissue culture supplies were from Gibco (Gaithersburg, MD). Fibroblasts were subcultured every 7–10 days with a split ratio of 1:4 after a 10-min incubation with 0.25% trypsin in Mg^{2+}- and Ca^{2+}-free D-PBS to detach the cells from the flask. Cells were used for experiments between passages 5 and 15 and were plated out at a concentration of 1×10^4 cells/cm^2 in 24-well dishes in 1 mL medium. At or near confluency, the day prior to experimentation, cells were changed to serum-free D-MEM/F12.

Intracellular triglyceride synthesis

For experiments, cells were incubated in D-MEM/F12 supplemented with 100 μM oleate: BSA (average specific activity: 100 dpm/pmol). To prepare oleate: BSA [9,10-^{3}H(N)]-oleic acid (DuPont-New England Nuclear, Mississauga, Canada) 10.0 Ci/mmol was diluted with cold oleate complexed to BSA in a 5:1 molar ratio as described by Van Harken, Dixon, and Heimberg (17). Test compounds were added at the indicated concentrations from working dilutions freshly prepared in D-PBS. In all cases, triglyceride synthesis was measured for 6 h. Triglyceride synthesis under these conditions is linear up to 24 h. After incubation for 6 h at 37°C, the cells were washed three times with 1 mL ice-cold D-PBS and extracted with two 1-mL volumes of heptane–isopropanol 3:2 (v/v). Lipid extracts were dried in a centrifuge-evaporator (Canberra-Packard, Canada), reconstituted in 100 μl chloroform–methanol 2:1 (v/v) and resolved by thin-layer chromatography (silica gel 150A, Whatman, England). Plates were developed in hexanes–ethyl ether–acetic acid 75:25:1 (v/v) with reference lipids run concurrently. The lipids were visualized with iodine vapor and the spots corresponding to triacylglycerol were scraped into scintillation vials containing 5 mL of scintillation fluid (Cytoscint-ES, ICN California) and counted by scintillation spectrometry (Beckman, CA). Cell proteins were dissolved on the dishes by addition of 1 mL of 0.1 N NaOH and measured by the method of Bradford (18) using a commercial kit (Bio-Rad, Richmond, CA).

Oleic acid (sodium salt), bovine serum albumin essentially fatty acid-free (BSA), 1-oleoyl-2-acetyl-*rac*-glycerol (OAG), staurosporine, 4β-phorbol 12-myristate 13-acetate (PMA), and bradykinin were purchased from Sigma (St. Louis, MO). 4α-Phorbol 12-myristate 13-acetate (4α-PMA) was purchased from Biolmol Research Laboratories (Plymouth Meeting, PA). Calphostin C and GF 109203X were from Calbiochem (San Diego, CA).

Intracellular diacylglycerol determination

Human skin fibroblasts were prepared as described above. Near confluency, cells were changed to 10% FCS D-MEM/F12 supplemented with 0.5 μCi/mL of [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (218 Ci/mmol) (DuPont-New England Nuclear, Mississauga, Canada) for 48 h. For the experiment, cells were exposed to test agents (bradykinin, ASP, or PMA) at the indicated concentrations and for the indicated times. After incubation, the cells were placed on ice and immediately washed three times with 1 mL ice-cold D-PBS and extracted with two 1-mL volumes of heptane–isopropanol 3:2 (v/v). A portion of the lipid extracts was counted directly. The remaining lipid extracts were treated as described above to separate the individual lipids and the spots corresponding to diacylglycerol were quantitated. Results are expressed as the percentage radioactive diacylglycerol (DPM) generated per total lipid extract (DPM). Results are compared to the basal diacylglycerol generation in control (untreated) cells which is taken as 100%.

Protein kinase C translocation and activity

Human skin fibroblasts were grown in T-75 flasks. Near confluency, the day prior to experimentation, cells were changed to serum-free D-MEM/F12. For experiments, cells were changed to fresh serum-free medium

supplemented with PMA or ASP at the indicated concentrations and time periods. After the incubation period, cells were immediately washed three times with ice-cold D-PBS, detached with a cell scraper, transferred to microcentrifuge tubes on ice, and pelleted by centrifugation. Cells were fractionated as follows. The cell pellet was homogenized in 0.5 ml of ice-cold extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 25 μg/ml each aprotinin and leupeptin) and centrifuged in an airfuge (Beckman Instruments, LaJolla, CA) at 100,000 *g* for 30 min. The resulting supernatant contained the soluble cytosolic protein kinase C. The pellet was resolubilized in extraction buffer with 0.5% Triton X-100 to solubilize particulate membrane protein kinase C. Protein kinase C activity was measured using a commercial kit (Gibco BRL, Gaithersburg, MD). Yasuda et al. (19) have reported that a synthetic peptide from myelin basic protein (MBP) can act as a specific substrate for protein kinase C and an N-terminally acetylated form of this peptide is used in this assay. The regulatory domain of protein kinase C contains a pseudosubstrate region that is involved in maintaining the enzyme in the inactive state. Antibodies directed against the pseudosubstrate sequence activate protein kinase C and peptides containing this sequence are potent inhibitors. The pseudosubstrate peptide PKC(19-36) has a K_i of 0.15 μM with essentially no inhibition of other protein kinases (20). Therefore, in this assay, protein kinase C specificity is confirmed by using the MBP synthetic peptide as substrate and using the protein kinase C pseudosubstrate inhibitor peptide as a potent inhibitor for this substrate. The difference between activity in the presence versus absence of the inhibitor is the specific PKC activity.

Data analysis

Data is reported as mean of "n" experiments (with all determinations performed in triplicate within each experiment) ± standard error of the mean. Statistical significance was determined by a one-tailed Student's *t*-test and set at $P < 0.05$ where pNS is not significant.

RESULTS

The focus of this study was to determine the second messenger signalling pathway of ASP. Based on our previous and present work on ASP, we have modified our isolation protocol substantially. The present method used is described in detail in Methods and is summarized in Table 1. This purification method results in greater total yields of ASP in a shorter time period. On average, 33 mg of ASP can be obtained per preparation which is much greater than the 0.5 mg yield reported in our original publication (21).

The strategy to determine the potential second messenger signalling pathway for ASP was as follows. First, known stimulators of specific second messenger pathways were added to the human skin fibroblast cell model to assess their capacity to stimulate triglyceride synthesis in a manner analogous to ASP. In all cases, human skin fibroblasts were incubated in serum-free medium containing 100 μM [^{3}H]oleate complexed to BSA and the indicated additions. Triglyceride synthesis (TGS) was measured over a 6-h period, a time period that we have previously shown to be in the linear range for TGS in these cells (21). Next, in those cases where the results were positive, we assessed whether ASP was acting through the test pathway in three ways: *i)* additivity of the ASP effect on triglyceride synthesis with positive compounds; *ii)* inhibition of the ASP effect on triglyceride synthesis using known inhibitors of these pathways; and finally *iii)* determination of cellular biochemical changes associated with the signalling pathway of interest.

Stimulators of second messenger pathways

The possible involvement of protein kinase C (PKC) as a mediator of ASP action was first investigated by determining the effect of 4β phorbol 12-myristate 13-acetate (PMA), a known stimulator of protein kinase C (22), on triglyceride synthesis. **Figure 1** demonstrates

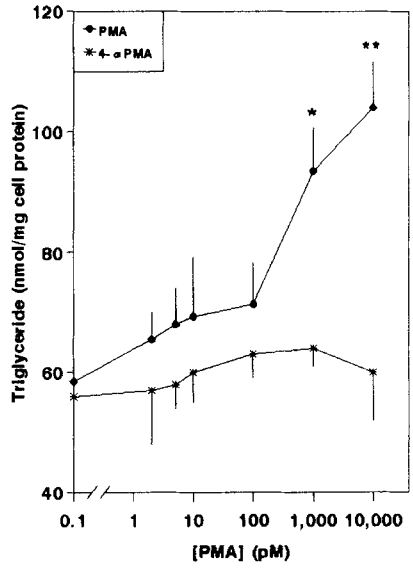

Fig. 1. Effect of phorbol esters on triglyceride synthesis. Human skin fibroblasts were incubated at 37°C with media containing 100 μM [^{3}H]oleate and various concentrations of either 4α-phorbol 12-myristate 13-acetate (4α-PMA) or 4β-phorbol 12-myristate 13-acetate (PMA). The incorporation of [^{3}H]oleate into intracellular triglycerides was measured after a 6-h incubation as described in the Methods section. Data are expressed as nmol oleate incorporated into intracellular triglyceride per mg of cell protein per 6 h ± SEM. Each point represents the average of four experiments with each determination performed in triplicate; $*P < 0.01$; $**P < 0.0005$.

that PMA is a potent stimulator of triglyceride synthesis at concentrations known to activate PKC in cells (23). The maximal increase in TGS was 178% ± 12% of basal ($P < 0.0005$) at 10 nM PMA. By contrast, 4α-PMA, an isomer of PMA which does not activate PKC (23), had no effect on triglyceride synthesis, confirming the specificity of PMA action on PKC (Fig. 1).

OAG (1-oleoyl-2-acetyl-*rac*-glycerol) is an analog of diacylglycerol and is also a potent stimulator of protein kinase C (24). As shown in **Fig. 2,** increasing concentrations of OAG also increased triglyceride synthesis. A maximal effect was achieved at a concentration of 250 μM (181% ± 11%, $P < 0.025$). OAG did not appear to be a substrate itself for acylation as there was no radioactive lipid component that migrated between authentic OAG and authentic triglyceride (triolein) on thin-layer chromatography as would be expected if OAG was acylated to generate a pseudo-triglyceride.

In 3T3 clonal murine cell line and rat adipocyte precursor cells, increases in cAMP have been shown to be essential in order to induce differentiation and to increase triglyceride accumulation (25, 26). It is also well recognized that cAMP plays a role in activation of hormone-sensitive lipase (27). Because cAMP is important in both lipogenesis and lipolysis, we tested whether ASP might act via this pathway. Cholera toxin increases cAMP (28, 29) as well as activating phospholipase pathways (30), generating arachidonic acid (31) and inhibiting PIP kinase (32). Increasing concentrations of cholera toxin increased triglyceride synthesis up to 335% ± 112% of basal ($P < 0.0025$) at 100 ng/ml (data not shown). In contrast, pertussis toxin, which ADP-ribosylates Gi resulting in persistent inactivation of Gi, had no effect on triglyceride synthesis (data not shown). Moreover, the beta subunit of cholera toxin, which has been shown to interact with plasma membrane GMI ganglioside (33), also had no effect on triglyceride synthesis (data not shown). Other compounds that cause an increase in intracellular cAMP (isobutylmethyl xanthine or forskolin) or the addition of permeable analogs of cAMP (dibutryl cAMP or 8-bromo cAMP) had no effect on triglyceride synthesis, suggesting that protein kinase A does not play a role in ASP-mediated increases in triglyceride synthesis (data not shown).

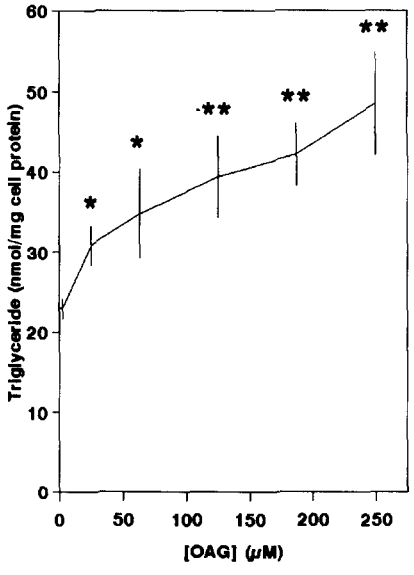

Fig. 2. Effect of oleoyl-acetyl glycerol (OAG) on triglyceride synthesis. Human skin fibroblasts were incubated at 37°C with media containing 100 μM [^{3}H]oleate and various concentrations of oleoyl-acetyl-glycerol (OAG). The incorporation of [^{3}H]oleate into intracellular triglycerides was measured at the end of the 6-h incubation time as described in the Methods section. Data are expressed as nmol oleate incorporated into intracellular triglyceride per mg of cell protein per 6 h ± SEM. Each point represents the average of four experiments with each determination performed in triplicate; *$P < 0.05$; **$P < 0.025$.

Additivity of TGS stimulators with ASP

The results demonstrate that compounds that increase protein kinase C activity (PMA and OAG in Figs. 1 and 2) increase triglyceride synthesis. We therefore tested the additivity of these compounds on the ASP-mediated increase in triglyceride synthesis. The cholera toxin effect was additive to that of ASP, even at concentrations where both were individually maximally active, suggesting that ASP and CTX act via different mechanisms (data not shown). As shown in **Fig. 3,** the effect of PMA on triglyceride synthesis was not additive to that of ASP. As ASP concentration is increased to 25 μg/mL, triglyceride synthesis increases to a maximum of 196% ± 11%. In the presence of 1 nM PMA, triglyceride synthesis is also increased (174% ± 12%) compared to control (no PMA, no ASP) $P < 0.025$, but with the addition of increasing ASP (up to 25 μg/mL) and 1 nM PMA triglyceride synthesis was not significantly greater than the maximal level of triglyceride synthesis obtained with ASP alone (ASP 25 μg/mL) + PMA (1 nM): 225% ± 21%, P = NS vs. ASP alone). This supports the hypothesis that the activation of PKC is integral to ASP action.

Effect of protein kinase C inhibitors on ASP stimulation of triglyceride synthesis

To examine further the involvement of PKC as a mediator of ASP action, we tested the ability of a number of protein kinase C inhibitors to inhibit the ASP-induced stimulation of triglyceride synthesis. As shown previously in Figs. 1 and 3, PMA stimulated triglyceride synthesis to the same extent as ASP. For the following experiments, cells were stimulated maximally with either 25 μg/mL ASP or 1 nM PMA. In all cases (**Fig. 4,** all three panels), the maximal stimulation of triglyceride synthesis produced by PMA was comparable to that produced by ASP (193% ± 14 PMA, $P < 0.0005$

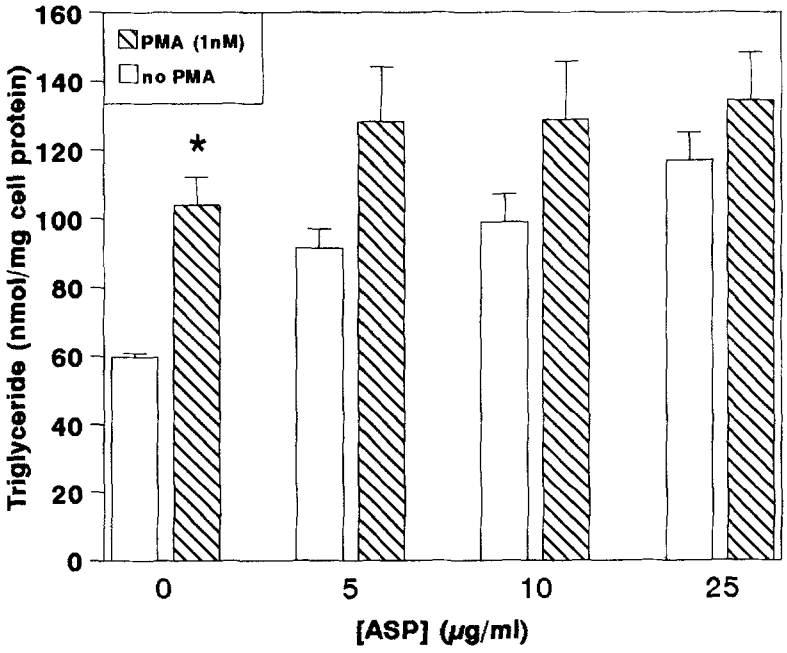

Fig. 3. Effect of simultaneous addition of PMA and ASP on triglyceride synthesis. Human skin fibroblasts were incubated at 37°C in media containing 100 μM [^{3}H]oleate with either no addition or 1 nM PMA and various concentrations of ASP. The incorporation of [^{3}H]oleate into intracellular triglycerides was measured over a 6-h incubation time as described in the Methods section. Data are expressed as nmol oleate incorporated into intracellular triglyceride per mg of cell protein per 6 h ± SEM. Each point represents the average of three experiments with each determination performed in triplicate; *$P < 0.025$ with PMA vs. control (no PMA, no ASP).

vs. basal and 173% ± 10 ASP, $P < 0.0025$ vs. basal, P NS between ASP and PMA). We first tested the effects of staurosporine, a potent inhibitor of PKC which is 10 times more effective at inhibiting PKC (K_i 0.7 nM) than protein kinase A (K_i 7 nM in cell extracts) (34). The results of these experiments are shown in Fig. 4, left panel. Staurosporine was found to inhibit both the ASP and PMA stimulation of triglyceride synthesis in human skin fibroblasts in a concentration-dependent manner. The maximal effect obtained was 59% ± 4.9% inhibition

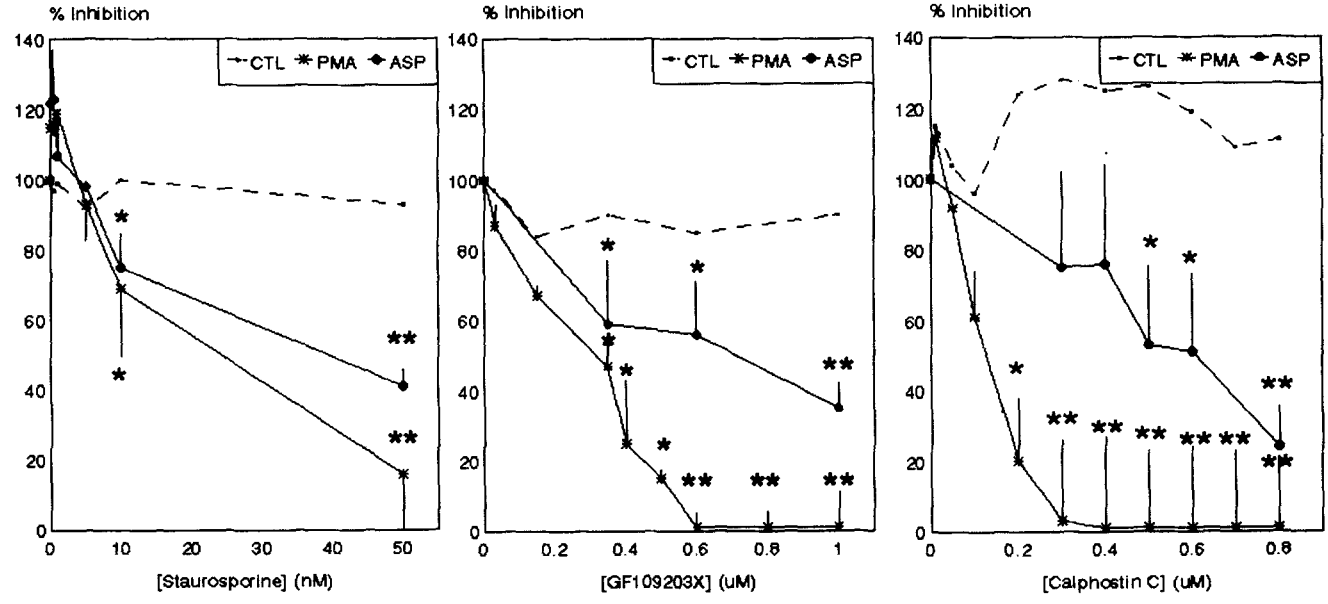

Fig. 4. Effect of staurosporine, GF109203X and calphostin C on ASP- and PMA-stimulated triglyceride synthesis. Human skin fibroblasts were incubated at 37°C in media containing 100 μM [^{3}H]oleate with either no addition (control), 25 μg/mL ASP or 1 nM PMA and increasing concentrations of staurosporine (left panel, 2 experiments), GF109203X (middle panel, 4 experiments), or calphostin C (right panel, 4 experiments). The incorporation of [^{3}H]oleate into intracellular triglycerides was measured over a 6-h incubation time as described in the Methods section and calculated per mg cell protein. Data are expressed as % inhibition of the ASP or PMA stimulated response ± SEM where the average ASP stimulation (without any inhibitor present) was 173% ± 10%, ($P < 0.0025$) and the average PMA stimulation was 193% ± 14% ($P < 0.0005$ for all 10 experiments). For clarity, error bars are not included for control (CTL) incubations with inhibitors; however, on average, basal values of TG synthesis did not vary by more than 10% for any inhibitor at any concentration. Each point represents the average of experiments with each determination performed in triplicate; *$P < 0.05$; **$P < 0.005$.

of the ASP effect ($P < 0.005$) and 84% ± 16% inhibition of the PMA effect ($P < 0.005$). Staurosporine had no effect on basal triglyceride synthesis (Fig. 4, left panel).

GF109203X (bisindolylmaleimide) is a protein kinase C inhibitor structurally similar to staurosporine but is highly selective for PKC inhibition (K_i 0.014 μM vs. a K_i of 2 μM for protein kinase A in cell extracts) (35). GF 109203X acts as a competitive inhibitor of the ATP binding site on PKC. As shown in Fig. 4, center panel, addition of this compound to the fibroblasts resulted in effective inhibition of both the ASP- and PMA-stimulated triglyceride synthesis to a maximum of 65% ± 7 ASP, $P < 0.005$ and 99% ± 10 PMA, $P < 0.005$ inhibition.

Calphostin C is a highly specific inhibitor of PKC that interacts with the regulatory domain and competes for the binding of diacylglycerol and phorbol esters (K_i 0.05 μM for PKC vs. K_i > 50 μM for protein kinase A in cell extracts) (36). As shown in Fig. 4, right panel, calphostin C effectively inhibited the ASP stimulation of triglyceride synthesis by 76 ± 11%, $P < 0.005$. A comparable inhibition of the PMA-induced stimulation was also achieved (up to 99 ± 20% inhibition at 0.8 μM calphostin C, $P < 0.005$). These data establish that a number of protein kinase C inhibitors that act through different mechanisms were all effective at reducing the ASP stimulation of triglyceride synthesis.

We also attempted to down-regulate protein kinase C with prolonged incubation with PMA and then reassess ASP stimulation of triglyceride synthesis. Although there was less stimulation by ASP in PMA-treated cells (expressed as percent stimulation), the PMA treatment of the cells itself caused basal triglyceride synthesis to increase at least twofold, making the data difficult to interpret (data not shown).

Biochemical markers of activation

Protein kinase C activation is accompanied by a number of intracellular changes. Increases in intracellular diacylglycerol (DAG), a second messenger, have been demonstrated in fibroblasts, adipocytes, and other cells stimulated by the peptide mitogen bradykinin which activates protein kinase C (37–39). Intracellular DAG increases are often the result of activation of phospholipase C or of phospholipase D coupled to phosphatidate phosphohydrolase. Translocation of protein kinase C from the intracellular cytosolic fraction to the plasma membrane particulate fraction is a marker of PKC activation and has been demonstrated in fibroblasts and adipocytes (39, 40). Activation of protein kinase C results in phosphorylation of a number of substrates on serine/threonine residues including phosphorylation of specific substrates such as myelin basic protein and MARCKS protein (19). We therefore examined the following intracellular changes: diacylglycerol generation and protein kinase C translocation in ASP-stimulated human skin fibroblasts.

Intracellular diacylglycerol release was measured after equilibrium labeling of human skin fibroblasts with arachidonic acid. Bradykinin has been shown to produce acute increases in diacylglycerol in human skin fibroblasts in association with stimulation of protein kinase C (37, 38). Human skin fibroblasts were plated out in 10% FCS in MEM as described in Methods. Near confluence, cells were changed to a 10% FCS medium supplemented with 0.5 μCi/mL [^{3}H]arachidonic acid for 48 h. Cells were changed to serum-free medium for 6 h, followed by stimulation with ASP or bradykinin for times ranging from 0.5 to 90 min. Intracellular radiolabeled diacylglycerol release was determined. As shown in **Fig. 5** (right panel), bradykinin (200 nM) produced a biphasic response, as previously reported (37), causing a 149% ± 19% increase in diacylglycerol generation at 0.5 min, $P < 0.05$, and at 20 min (149 ± 11% increase, $P < 0.025$). Similarly, ASP caused a time-dependent release of intracellular diacylglycerol which peaked initially at 5 min (142% ± 8%, $P < 0.025$) and then again at 30 min (164% ± 17%, $P < 0.025$). The first peak returned rapidly to baseline (by 10 min) whereas the second peak was of greater magnitude and its duration was much longer. Although the diacylglycerol increase peaked at 30 min in the second phase, in fact, it was increased significantly over an extended period from 20 min (120% ± 6%, $P < 0.05$) to 60 min (125% ± 6%, $P < 0.05$). In contrast, addition of PMA to the cells did not produce an early peak in diacylglycerol, but did produce a later diacylglycerol increase that peaked at 30 min (data not shown).

We have also quantified the translocation of protein kinase C from the soluble cytosolic compartment to the particulate plasma membrane surface. Cells were incubated for various times with ASP or with PMA. Incubations were terminated and the cells were lysed with ice-cold buffers and fractionated into membrane and soluble fractions. Protein kinase C activity was determined in the cell homogenates and in both cell fractions using a commercial assay as described in Methods. In the presence of 50 nM PMA, total cellular protein kinase C activity increased by 19% ± 22% (pNS) after a 15-min incubation. ASP increased total protein kinase C activity by 35% ± 12%, $P < 0.05$, reaching a maximum at 30–40 min incubation. The results for ASP translocation of protein kinase C from cytosolic to membrane fractions are shown in **Fig. 6,** left panel. At time zero, 78% ± 12% of the cellular total protein kinase C activity was in the cytosolic compartment and 22% ± 12% was in the plasma membrane compartment. ASP caused a time-dependent translocation of protein kinase C from the cytosol to the membrane reaching a maximal translocation at 30–40

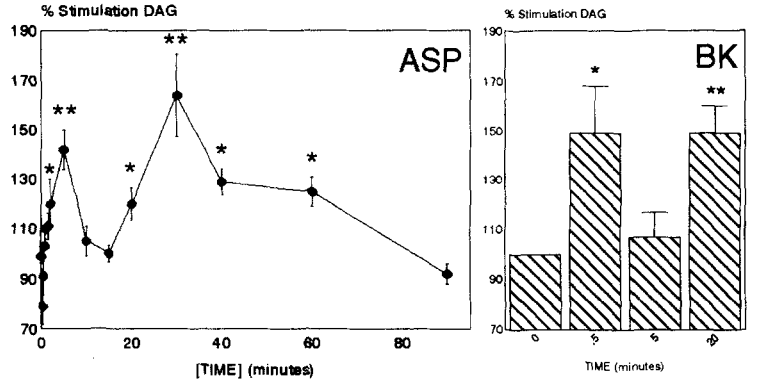

Fig. 5. Effect of ASP and bradykinin on intracellular diacylglycerol accumulation. Human skin fibroblasts were incubated for 48 h at 37°C with media containing 0.5 μCi/mL [^{3}H]arachidonic acid. The cells were changed to a serum-free medium for 6 h and were then challenged with 25 μg/mL ASP or 200 nM Bradykinin (BDK) for the indicated time periods. The data was calculated as radioactivity (dpm) intracellular [^{3}H]diacylglycerol generation per dpm total ^{3}H-labeled lipid extract. Results are expressed as percent change in labeled diacylglycerol as compared to basal (100%) for an average of 9 experiments (ASP) or 4 experiments (BK) ± SEM; $*P < 0.05$; $**P < 0.025$.

min incubation. The translocation was transient and by 80 min, protein kinase C activity had returned to the basal state. PMA-mediated protein kinase C translocation was also time-dependent and more rapid than ASP, reaching a maximum at 15 min of incubation, thereafter returning to basal levels consistent with published data (40) (data not shown). Preliminary experiments over a short time period (0–15 min) did not demonstrate a rapid (less than 10 min) effect of ASP on protein kinase C translocation (data not shown). The maximal translocation activity of ASP is compared to PMA in Fig. 6, right panel. After a 15-minute incubation with PMA, translocation was maximal and 69% ± 10% of total activity was found in the plasma membrane ($P < 0.0025$). The maximal effect of ASP was comparable to that achieved with PMA; 54% ± 15% of total protein kinase C activity was found in the plasma membrane ($P < 0.005$), although the average time to reach this was longer, 35 ± 7 min.

DISCUSSION

The triglyceride content of an adipocyte is determined by the balance between synthesis and lipolysis. Lipolysis is initiated by the action of hormone-sensitive lipase, the enzyme that catalyzes the rate-determining step in this pathway (41). The molecular events responsible for regulation of triglyceride hydrolysis in adipo-

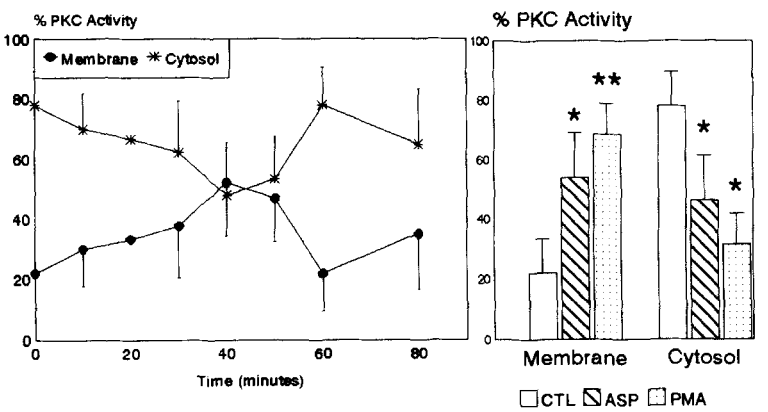

Fig. 6. Effect of ASP and PMA on intracellular protein kinase C translocation. At confluency, human skin fibroblasts were changed to a serum-free medium for 6 h and were then challenged with 50 nM PMA (for 15 min) or 25 μg/mL ASP for the indicated time periods. Cells were then washed, homogenized in extraction buffer, and the soluble cytosqlic protein kinase C was separated from the particulate membrane-bound protein kinase C by ultracentrifugation. The particulate membrane preparation was resolubilized and protein kinase C activity for both soluble and membrane fractions was measured as described in Methods. Left panel: results are expressed as the percent distribution of protein kinase C activity between the cytosolic and membrane fractions after incubation with ASP for the indicated times. Right panel: percent distribution of protein kinase C activity for the maximal ASP-induced translocation (25 μg/mL ASP, average 36.7 ± 7.2 min) and the PMA-induced translocation (50 nM PMA, 15 min, for n = 6 experiments); $*P < 0.05$; $**P < 0.0025$ vs. control (CTL).

cytes are well characterized and involve ligand-dependent activation of β-adrenergic receptors on the extracellular surface of the adipocyte cell membrane. The transduction of this coupling is mediated intracellularly by the GTP-binding protein Gs which, when activated, stimulates membrane-bound adenylate cyclase to increase cAMP concentrations within the cell. This change in cAMP activates protein kinase A which phosphorylates, and thus activates, hormone-sensitive lipase (27). In contrast, the regulation of triglyceride synthesis in adipocytes has yet to be described at a molecular level, and as noted at the outset, until recently there has been little evidence to suggest that this pathway is physiologically regulated. This study addresses that issue with regard to the mechanism of action of ASP.

The results demonstrate that ASP stimulation of triglyceride synthesis is effected through a protein kinase C-dependent mechanism. The evidence pointing to this is as follows. *i)* The effect of ASP on triglyceride synthesis could be mimicked by PMA and OAG, both stimulators of protein kinase C; *ii)* in the presence of saturating concentrations of PMA, addition of ASP does not increase triglyceride synthesis above that of ASP alone; *iii)* staurosporine, calphostin C, and GF109203X, specific inhibitors of protein kinase C at either the catalytic or regulatory sites, inhibited the ASP effect on triglyceride synthesis; *iv)* addition of ASP increases intracellular diacylglycerol, the physiological activator of protein kinase C; and lastly, *v)* ASP stimulates time-dependent reversible translocation and activation of protein kinase C to the plasma membrane compartment. It should be noted that the physiological action of ASP is to cause increased triglyceride synthesis and glucose transport (5). If ASP is acting via a protein kinase C pathway it would not be surprising that PMA was capable of mimicking ASP action. In fact, the results presented here are consistent with previous reports demonstrating that phorbol esters stimulate triglyceride synthesis in rat adipocytes (8, 9) cultured fibroblasts (10) and other cells (11) and glucose transport in isolated rat adipocytes and cultured myocytes (12, 13).

The repression of the ASP-stimulated triglyceride synthesis by diverse protein kinase C inhibitors confirms that ASP acts via a protein kinase C pathway. Clearly, however, the inhibitors are not as effective in reducing ASP action as they are at inhibiting PMA stimulation. Part of this differential effect may be related to the protein kinase C isozyme distribution. Up to nine members of the protein kinase C family have been identified (42) with at least four isozymes (both Ca^{2+}-dependent and -independent) recognized in human skin fibroblasts (43). Isozymes may respond differentially to stimulation by PMA or ASP or to inhibition by staurosporine, calphostin, and GF109203X. More importantly, it should be noted that although protein kinase C is stimulated by diacylglycerol (generated endogenously by ASP or supplied exogenously as PMA in the incubation media), protein kinase C activity is also stimulated by other molecules including other lipids. Thus, fatty acids, fatty acyl CoA, phosphatidyl serine, and Ca^{2+} all enhance protein kinase C activity and some (or all) of these factors may be increased by ASP cell-targeted actions in addition to ASP-induced increases in diacylglycerol (42, 44, 45).

Diacylglycerol is generated through phospholipase C cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce 1,4,5-triphosphate (IP_3) and diacylglycerol. Alternatively, phospholipase C may also act on phosphatidylcholine to generate diacylglycerol and phosphocholine. An alternate pathway of diacylglycerol generation involves the sequential actions of phospholipase D and phosphatidate phosphohydrolase on phosphatidylcholine resulting in production of diacylglycerol. It has been shown (here and in published studies) that bradykinin action on fibroblasts is biphasic, with the first diacylglycerol peak resulting from phospholipase C action on phosphoinositide hydrolysis (with concomitant increases in IP_3 and decreases in PIP and PIP_2) whereas the later plateau phase of diacylglycerol production emanates from phosphatidylcholine breakdown (37, 46) with potential contribution from both phospholipase C and phospholipase D coupled to phosphatidate phosphohydrolase (47). In contrast, PMA stimulation of protein kinase C translocation was rapid and peaked by 15 min whereas diacylglycerol production was slower and produced only a single peak at the later time point. Phorbol esters have been shown to stimulate phosphatidylcholine breakdown and it has been proposed that rapid protein kinase C activation after initial phosphoinositide hydrolysis may contribute to a positive feedback loop to enhance phosphatidylcholine breakdown and prolonged diacylglycerol generation (48, 49).

It is interesting that ASP produces a biphasic effect on diacylglycerol generation. Although in the present studies with ASP, it is not clear which phospholipase is responsible for diacylglycerol generation, the biphasic nature suggests that diacylglycerol accumulation results from the breakdown of different phospholipids. In the experiments on the ASP effect on protein kinase C translocation and activation, we did not see an early, rapid and transient increase in protein kinase C, but only a later increase at 30–50 min, which peaked at approximately 40 min. It may well be, however, that the technical difficulties of measuring protein kinase C activity in cell extracts that have to be homogenized and separated by centrifugation precludes measuring minor transient ASP effects on protein kinase C translocation at early time points. However, the second peak of diacyl-

glycerol generation was more pronounced and prolonged and this was clearly accompanied by translocation and activation of protein kinase C.

Overall then, the pattern of ASP response is a biphasic diacylglycerol generation with a peak of protein kinase C translocation and activity occurring at the same time as the second diacylglycerol peak. One possible scenario is that ASP interacts with the cell membrane activating a phospholipase and generating intracellular diacylglycerol. This increase in diacylglycerol activates and translocates protein kinase C which further enhances phospholipase activity increasing phospholipid breakdown and diacylglycerol generation.

How might ASP cause increases in triglyceride synthesis via protein kinase C? As ASP stimulates glucose transport and fatty acid acylation, ASP might increase the level of the diacylglycerol intermediate in the triglyceride biosynthetic pathway and this in itself might activate protein kinase C. This is not likely as we have previously shown that diacylglycerol constitutes only a very small percentage (5%) of total oleate incorporation into lipids in human skin fibroblasts and that in the presence of ASP, there is no significant stimulation of ASP on diacylglycerol production (16% increase in diacylglycerol vs. a 110% increase in triglyceride) with overall a slight decrease in the distribution of diacylglycerol (4%) (21). Additionally, studies in crude microsomal preparations demonstrated no ASP effect on phosphatidate phosphohydrolase (the enzyme which produces diacylglycerol in the triglyceride biosynthetic pathway) but a pronounced effect on diacylglycerol acyltransferase, which would enhance product formation (triglyceride) at the expense of the precursor diacylglycerol (16). Previous findings in rat hepatic microsomes have shown that the activities of glycerol-3-phosphate acyltransferase, the enzyme catalyzing the first acylation reaction in triglyceride synthesis, and of diacylglycerol acyltransferase, the enzyme catalyzing what has been suggested as the rate-determining step in the triglyceride synthetic pathway, could both be modulated experimentally in a manner consistent with a phosphorylation/dephosphorylation-dependent activation (50, 51). As we have previously demonstrated that ASP markedly enhances the activity of diacylglycerol acyltransferase (6), this enzyme may very well be the site modulated by ASP-dependent protein kinase C activation. Stimulation of protein kinase C may activate a phosphatase resulting in dephosphorylation of and activation of triglyceride synthetic enzymes as has been demonstrated in other systems (52, 53).

Insulin also affects triglyceride balance in adipocytes. Its best defined action is stimulation of phosphodiesterase which breaks down cAMP and thus inhibits lipolysis (54, 55). Insulin also stimulates glucose transport through translocation of glucose transporters and perhaps through changes in intrinsic activity of the glucose transporter (56–58). However, in human adipocytes, the effect of insulin on triglyceride synthesis is only moderate in contrast to that seen in rat adipocytes (59). It is important to note that we have previously shown that the effect of ASP on glucose transport and triglyceride synthesis is independent of, and additive to, the effects of insulin in human skin fibroblasts (5). Therefore, although ASP and insulin act independently, they both do so through a cell interaction which markedly affects second messenger cell signalling processes.

Although much work remains to be done to elucidate the complete pathway of the molecular mode of action of ASP, these data indicate that the signal transduction pathway of ASP is, at least in part, mediated through protein kinase C. It is interesting to speculate how this may relate to the reduced ASP responsiveness demonstrated in HyperapoB. HyperapoB patients are characterized by an increased production rate of hepatic apoB-100 lipoproteins (60) and this is often associated with a decreased efficiency of triglyceride clearance in peripheral tissues such as adipose tissue (61, 62). We have proposed that this may relate to impaired peripheral action of ASP as a diminished response to ASP in cultured human skin fibroblasts from a subset of patients with HyperapoB has been observed (7, 63). In light of the present study demonstrating that ASP action is mediated by a protein kinase C mechanism, future studies will examine this pathway in HyperapoB patients. It remains to be seen whether the defect in ASP response in HyperapoB cells is primarily at the cell-receptor interaction or related to defective intracellular signalling or intracellular triglyceride synthesis.

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