Mechanisms Involved in the Regulation of Free Fatty Acid Release from Isolated Human Fat Cells by Acylation-stimulating Protein and Insulin*

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The effects of acylation-stimulating protein (ASP) and insulin on free fatty acid (FFA) release from isolated human fat cells and the signal transduction pathways to induce these effects were studied. ASP and insulin inhibited basal andnorepinephrine-induced FFA release by stimulating fractional FFA re-esterification (both to the same extent) and by inhibiting FFA produced during lipolysis (ASP to a lesser extent than insulin). Protein kinase C inhibition influenced none of the effects of ASP or insulin. Phosphatidylinositol 3-kinase inhibition counteracted the effects of insulin but not of ASP. Phosphodiesterase 3 (PDE3) activity was stimulated by ASP and insulin, whereas PDE4 activity was slightly increased by ASP only. Selective PDE3 inhibition reversed the effects of both ASP and insulin on fractional FFA re-esterification and lipolysis. Selective PDE4 inhibition slightly counteracted the ASP but not the effect of insulin on fractional FFA re-esterification and did not prevent the action of ASP or insulin on lipolysis. Thus, ASP and insulin play a major role in regulating FFA release from fat cells as follows: insulin by stimulating fractional FFA re-esterification and lipolysis and ASP mainly by stimulating fractional FFA re-esterifica-

The release of free fatty acids (FFA) from fat cells plays an important role in the energy homeostasis of the body. This is mainly the result of two processes occurring simultaneously in the fat cell, lipolysis and FFA re-esterification. During lipolysis, triglyceride molecules are hydrolyzed to free fatty acids (FFA) and glycerol, which are released from the fat cell. Some of the FFA formed during lipolysis can be re-esterified to triglycerides (1, 2), whereas little or no glycerol is re-utilized by the fat cells (3). This pathway of lipolysis and FFA re-esterification forms an important cycle for energy turnover, allowing the fat cell to respond rapidly to changes in peripheral requirements for FFA (for discussion see Refs. 1 and 4–6).

Two types of FFA re-esterification in fat cells can be recognized (4). Primary re-esterification is the total amount of FFA that is re-esterified during a given situation and reflects the triglyceride synthesis capacity of the fat cells (7). Fractional re-esterification is the proportion of FFA re-esterified in relation to the amount of FFA formed by lipolysis in fat cells (4). The latter constitutes a futile cycle, energy-rich FFA first being formed by lipolysis of triglycerides and then synthesized to triglycerides without the achievement of any net biochemical work.

The mechanism by which the release of FFA from fat cells is regulated is only partly known; insulin has been shown to inhibit lipolysis in a phosphatidylinositol 3-kinase (PI3K) and phosphodiesterase 3B (PDE3)-dependent manner (8) and to stimulate fractional re-esterification (9), resulting in inhibition of FFA release. However, little is known about the signal transduction pathways involved in the action of insulin on FFA re-esterification, although PI3K seems to be involved (10).

Another protein with a potentially important effect on FFA release from human fat cells is acylation-stimulating protein (ASP) (for a review see Ref. 11). Human fat cells synthesize and secrete three proteins in the complement family as follows: the third component of complement (C3), factor D (adipsin), and factor B. They interact to form C3a-des-Arg which is identical to ASP (12–16). It has been shown that ASP is a potent stimulator of triglyceride synthesis in human skin fibroblasts and fat cells (16–17). After interaction with a specific membrane receptor (18), it triggers a protein kinase C (PKC) pathway (19), resulting in two effects. First, ASP increases glucose transport by promoting the translocation of glucose transporters from an intracellular pool to the plasma membrane (20–22). The intracellular glucose can be used as the backbone of the triglyceride molecule, glycerol 3-phosphate. Second, ASP increases the activity of diacylglycerol acyltransferase, the enzyme catalyzing the final and rate-limiting step in triglyceride synthesis (23). These effects of ASP are additive to and independent of those observed with insulin (20, 24). The action of ASP on FFA
release from fat cells, on the other hand, is unknown.

The first aim of the present study was to determine the effect of ASP on FFA produced and re-esterified during lipolysis in isolated human subcutaneous fat cells and to compare these effects with those of insulin. The second aim was to investigate which intracellular signaling pathways for ASP and insulin control their effects on FFA release. One focus was on PKC and PI3K which are reported to be linked to the known effects of ASP and insulin, respectively, in fat cells. A second focus was on signaling downstream of PKC and PI3K. For the latter purpose, the roles of PDE3 and PDE4 were examined. These PDE subtypes are present in fat cells (25) where they regulate lipolysis by modifying intracellular cyclic AMP levels. PDE3 is known to be the key enzyme involved in the antilipolytic action of insulin (8). Our hypothesis was that PDE3 and other PDEs might regulate hormone effects on FFA re-esterification as well.

MATERIALS AND METHODS

Subjects—The study group consisted of 36 subjects who underwent elective open abdominal surgery for non-malignant disorders (gall-stone by cholecystectomy, and gastric banding for obesity). According to patient history, the subjects were healthy except for the surgical diagnosis. None was on regular medication or had undergone a recent weight reduction program. The subjects, who had fasted overnight, were anesthetized at 8.00 a.m., as described in detail (26). Before taking the fat biopsy, only saline was administered intravenously. Immediately after the abdominal wall had been opened, a specimen of subcutaneous fat tissue (2–5 g) was obtained from the surgical incision and immediately transported to the laboratory in saline at 37 °C.

Most patients at the hospital are at present operated on by laparoscopic methods, making them unsuitable for fat biopsy procedures. From the few remaining subjects undergoing open surgery, it was not possible to select for gender (10 men and 26 women), age (range: 25–68 years), or body mass index (BMI range: 21–90 kg/m²).

The study was approved by the Ethical Committee of the Karolinska Institute at Huddinge Hospital. It was explained in detail to all participants. All subjects gave their consent.

Preparation of ASP—The ASP was prepared at the Royal Victoria Hospital. It was isolated and purified from frozen human plasma (obtained from the local blood bank), as described previously (19). The purity of each ASP preparation was determined by ion spray mass spectrometry (95–99% pure), and the activity was tested through stimulation of [3H]oleic acid incorporation into intracellular triglycerides in human skin fibroblasts (19). ASP was frozen (−80 °C) in aliquots in phosphate-buffered saline (pH 7.2) containing 1 mg/ml bovine serum albumin and was shipped on dry ice to Huddinge Hospital, where all subsequent experiments took place. Insulin was not detected in the ASP stock solution after 10 min at 4 °C.

Enzyme-linked immunosorbent assay kit (Merckodia AB, Upssala, Sweden), which can detect insulin at a concentration as low as 1 µl.

Isolation of Fat Cells—Fat cells were isolated from the subcutaneous fat tissue specimen by collagenase treatment, as described (27). Briefly, diluted suspensions of fat cells (5–10,000 cells/ml) were incubated at 30 °C for 20 min in a total volume of 300 µl, containing 0.5 µ/liter fatty acid-free bovine serum albumin, 1 g/liter glucose, and 0.1 mg/liter ascorbic acid with or without insulin (0.1 nM) or ASP (5.6 µM). The fat cells were homogenized at room temperature with 10 strokes in a glass homogenizer in 1 ml of homogenization buffer containing 50 mM TES (pH 7.5), 250 mM sucrose, 1 mM EDTA, 8.3 mM MgCl₂, and 0.5 mM Hepes (pH 7.4), 0.1 mM EDTA, 8.3 mM MgCl₂, and 0.5 mM Hepes (pH 7.4), 0.1 mM EDTA, 8.3 mM MgCl₂, and 0.5 mM Hepes (pH 7.4), 0.1 mM EDTA, 8.3 mM MgCl₂, and 0.5 mM Hepes (pH 7.4). The total lipid content in the incubate was measured gravimetrically after organic extraction with heptane.

At the end of the incubation, a cell-free aliquot of the incubation medium was removed for glycerol release determination by a bioluminescence method (34). Another aliquot was used for the measurement of FFA release from fat cells, using a chemiluminescence method (35). FFA oxidation in this in vitro system is negligible (36), so the only expected outcomes for the FFA are either release from the fat cells during lipolysis or re-esterification. The glycerol release can be used as an accurate index of the amount of FFA released during lipolysis, as glycerol cannot be re-utilized by fat cells to any important extent (3). A correction of the expected outcomes for the FFA is negligible (36).

Statistical Analysis—Values are given as the mean ± S.E. Analysis of variance (ANOVA) and paired t test were used for statistical comparisons. A correction of the p value according to Bonferroni was made.
when it was appropriate. Correlations were analyzed using the linear regression method.

RESULTS

The Effect of ASP and Insulin on Release, Production during Lipolysis, and Primary Esterification of FFA—These experiments were performed in an identical fashion on 11 subjects. As shown in Fig. 1, ASP inhibited FFA release from fat cells incubated under basal conditions in a concentration-dependent way (ANOVA, F = 21, p < 0.0001). The minimum effective concentration was –6.3 log mol/liter (paired t test, p < 0.05) (corrected according to Bonferroni)). At the highest ASP concentration (–5.3 log mol/liter), FFA release was inhibited by ~85%. Norepinephrine (1 μM) doubled the rate of FFA release in comparison to basal incubation (paired t test p < 0.01). Also in combination with norepinephrine, ASP inhibited FFA release (ANOVA, F = 16, p < 0.0001). The minimum effective ASP concentration for this effect was –6 log mol/liter (paired t test (Bonferroni), p < 0.05) and at the highest ASP concentration, FFA release was inhibited by ~45%.

Insulin inhibited basal FFA release in a concentration-dependent fashion (Fig. 1, ANOVA, F = 20, p < 0.0001). The minimum effective concentration for this effect was –10 log mol/liter (paired t test (corrected according to Bonferroni), p < 0.05). At the highest insulin concentration of norepinephrine, insulin maintained its ability to inhibit FFA release in a concentration-dependent fashion (ANOVA, F = 20, p < 0.0001). The minimum effective insulin concentration for this effect was –10 log mol/liter insulin (paired t test (Bonferroni), p < 0.05) and at the highest insulin concentration, FFA release was inhibited by ~80%.

As shown in Fig. 2, ASP slightly inhibited the total amount of FFA produced during basal lipolysis (as calculated from glycerol release) in a concentration-dependent fashion (ANOVA, F = 10, p < 0.0001). The minimum effective ASP concentration was –6.3 log mol/liter (paired t test, p < 0.05 (Bonferroni)). At the highest ASP concentration (–5.3 log mol/liter), lipolysis-produced FFA was inhibited by ~30%. Norepinephrine (1 μM) almost doubled the rate of lipolysis in comparison to the basal state (p < 0.05). Also in combination with norepinephrine, ASP reduced the amount of FFA produced during lipolysis (ANOVA, F = 3, p < 0.01), but the effect was very small, i.e., lipolysis was inhibited by ~15% at the highest ASP concentration.

Insulin markedly inhibited the FFA production during basal lipolysis in a concentration-dependent fashion (Fig. 2, ANOVA, F = 14, p < 0.0001). The minimum effective concentration for this effect was –10 log mol/liter insulin (paired t test (Bonferroni), p < 0.05). At the highest insulin concentration (–9 log mol/liter), lipolysis was inhibited by ~60%. In the presence of 1 μM norepinephrine, insulin maintained its marked ability to inhibit lipolysis in a concentration-dependent fashion (ANOVA, F = 12, p < 0.0001). The minimum effective insulin concentration for this effect was –10 log mol/liter insulin (paired t test (Bonferroni), p < 0.05), and at the highest insulin concentration, the amount of FFA produced during lipolysis was decreased by ~60%.

The absolute rate of primary FFA esterification in the experiments described in Figs. 1 and 2 was calculated as the differ-
ence between total FFA produced by lipolysis (calculated from glycerol release) and the actual amount of FFA released from the cells. This rate was not influenced by ASP when fat cells were incubated in the basal state. The rates of primary esterification with and without the highest ASP concentration were similar: 2.92 ± 0.50 versus 2.82 ± 0.43 μmol/g lipid/2 h, p = not significant. Norepinephrine at 1 μM increased the absolute rate of primary FFA re-esterification (paired t test, 2.92 ± 0.50 versus 4.36 ± 0.54 μmol/g lipid/2 h, p < 0.05). Increasing concentrations of ASP did not change this effect of norepinephrine (paired t test, norepinephrine versus norepinephrine + ASP (~5.3 log mol/liter): 4.36 ± 0.54 versus 4.66 ± 0.56 μmol/g lipid/2 h, p = not significant). Insulin did not influence the absolute rate of primary FFA re-esterification significantly either, in the absence or presence of norepinephrine.

The fractional FFA re-esterification, which is the proportion of FFA re-esterified during lipolysis, increased markedly with increasing concentrations of ASP when fat cells were incubated in the basal state (Fig. 3, ANOVA, F = 4, p < 0.01). This stimulation was significant at −6.3 log mol/liter ASP (paired t test (Bonferroni), p < 0.01), and at the highest concentration, ASP increased fractional FFA re-esterification by ~40%. Norepinephrine alone at 1 μM decreased the fractional FFA re-esterification (paired t test, p < 0.01). In the presence of norepinephrine, ASP increased the fractional FFA re-esterification in a concentration-dependent manner (ANOVA, F = 5, p < 0.001). This stimulation started to be significant at −6.3 log mol/liter ASP (paired t test (Bonferroni), p < 0.05), and at the highest ASP concentration, fractional FFA re-esterification was increased by ~25%. Insulin also increased the fractional FFA re-esterification in a marked and concentration-depend-
in FFA release as compared with the effect of ASP or insulin alone. We also investigated the action of another PKC inhibitor, bisindoylmaleimide (GF 109203X), which is a competitive inhibitor of the ATP-binding site on PKC (41) at 1 and 5 μM. This blocker did not influence ASP or the action of insulin on FFA release in a significant way (values not shown).

The effect of wortmannin (PI3K inhibitor) on the action of ASP and insulin on FFA release was tested at a concentration of 0.1 μM. At this concentration wortmannin almost completely counteracts the effect of insulin on FFA release in isolated human fat cells (10). Wortmannin reversed the effect of insulin on FFA release almost completely (Fig. 4; paired t test, INS versus INS + wortmannin, p < 0.001). In contrast, it did not significantly influence the effect of ASP on FFA release (Fig. 4), indicating PI3K-dependent and PI3K-independent effects induced by insulin and ASP, respectively.

The results of the effects of enprofylline (a non-selective PDE inhibitor without any adenosine interactions), OPC 3911 (a selective PDE3 inhibitor), and rolipram (a selective PDE4 inhibitor) on FFA release are depicted in Fig. 5. The study with enprofylline was performed in an identical fashion on 10 subjects, and the studies with OPC 3911 and rolipram were performed on 9 subjects. Enprofylline (1 mM) and OPC 3911 (3 μM) reversed the effects of ASP and insulin on FFA release significantly (paired t test, ASP versus ASP + enprofylline, p < 0.001; INS versus INS + enprofylline; p < 0.001; ASP versus ASP + OPC 3911; p < 0.0001 and INS versus INS + OPC 3911; p < 0.0001). Enprofylline and OPC 3911 per se stimulated FFA release significantly (Fig. 5). Other concentrations of OPC 3911 were also tested (1 and 10 μM), and it was observed that OPC

### Table I

|                      | Basal | ASP    | INS     | ASP + INS |
|----------------------|-------|--------|---------|-----------|
| FFA release          |       |        |         |           |
| Basal                | 1.51 ± 0.28 | 0.23 ± 0.10<sup>a</sup> | 0.34 ± 0.07<sup>b</sup> | 0.03 ± 0.03 |
| NE                   | 3.04 ± 0.38 | 1.68 ± 0.29<sup>b</sup> | 0.79 ± 0.10<sup>b</sup> | 0.11 ± 0.05 |
| Lipolysis            |       |        |         |           |
| Basal                | 4.44 ± 0.75 | 3.05 ± 0.45<sup>c</sup> | 2.55 ± 0.33<sup>c</sup> | 2.16 ± 0.30 |
| NE                   | 7.41 ± 0.87 | 6.33 ± 0.78<sup>c</sup> | 3.39 ± 0.30<sup>c</sup> | 3.30 ± 0.42 |
| Fractional re-esterification |       |        |         |           |
| Basal                | 0.67 ± 0.02 | 0.93 ± 0<sup>c</sup> | 0.87 ± 0<sup>c</sup> | 0.98 ± 0.02 |
| NE                   | 0.59 ± 0.02 | 0.74 ± 0.03<sup>b</sup> | 0.76 ± 0.03<sup>a</sup> | 0.97 ± 0.01 |

NS, not significant; n = 11. NE, norepinephrine (1 μM). FFA release and lipolysis were expressed as μmol/g of lipid/2 h. Fractional FFA re-esterification can vary between 0 (no re-esterification) and 1 (all FFA re-esterified).

<sup>a</sup> Values are p < 0.01.
<sup>b</sup> Values are p < 0.001.
<sup>c</sup> Values are p < 0.05.
3911 counteracted the effects of ASP and insulin on FFA release in a concentration-dependent fashion (data not shown). Rolipram (1 μM) did not alter the insulin effect on FFA release, whereas it slightly counteracted the effect of ASP (paired t test, ASP versus ASP + rolipram, p < 0.01). Higher concentrations of rolipram (3 and 100 μM) were also tested. The same pattern of effects as at 1 μM was observed (data not shown).

Effects of PDE Blockers on FFA Production during Lipolysis—The effects of the PDE inhibitors on FFA produced during lipolysis, as calculated from the values for glycerol release, are shown in Table II. For practical reasons it was not possible to perform all types of lipolysis experiments simultaneously in all individuals. In order to compare results from different sets of subjects, the results were expressed as a percentage of basal (i.e. no drug added) FFA produced during lipolysis. The mean values for basal lipolysis did not differ in a significant way between the different sets of experiments.

Enprofylline (1 mM) per se significantly stimulated FFA produced during lipolysis (paired t test, control versus enprofylline, p < 0.01). It also counteracted the effect of ASP and insulin on lipolysis (paired t test, ASP versus ASP + enprofylline, INS versus INS + enprofylline; p < 0.001).

OPC 3911 (3 μM) by itself increased FFA produced during lipolysis (paired t test, control versus OPC 3911; p < 0.01), and it reversed the effect of ASP and insulin on lipolysis (paired t test, ASP versus ASP + OPC 3911, p < 0.0001; INS versus INS + OPC 3911, p < 0.001).

Rolipram (1 μM) did not influence basal FFA produced during lipolysis nor did it counteract the effects of ASP and insulin on this process.

Norepinephrine (1 μM) stimulated FFA produced during lipolysis to a much higher extent than observed with enprofylline and to the same extent as observed with OPC 3911 (paired t test, NE versus enprofylline, p < 0.05). ASP or insulin inhibited norepinephrine-stimulated lipolysis (paired t test, NE versus NE + ASP, p < 0.05; NE versus NE + INS; p < 0.001). The addition of enprofylline to norepinephrine did not increase lipolysis above that observed with norepinephrine alone. On the other hand, ASP or insulin could not reverse norepinephrine-stimulated lipolysis when enprofylline was present.

Effects of PDE Blockers on Fractional FFA Re-esterification—These data are derived from the same experiments that are presented in Fig. 5. Since the absolute rate of re-esterification was not influenced by insulin or ASP, the investigations only include fractional re-esterification. The results of the effects of enprofylline, OPC 3911, and rolipram are depicted in Fig. 6. Enprofylline (1 mM) and OPC 3911 (3 μM) almost completely reversed the effects of ASP and insulin on fractional re-esterification (paired t test, ASP versus ASP + enprofylline, p < 0.01; INS versus INS + enprofylline; p < 0.05; ASP versus ASP + OPC3911; p < 0.0001 and INS versus INS + OPC3911; p < 0.05). Rolipram (1 μM) did not alter the insulin effect on fractional FFA re-esterification, whereas it slightly counteracted the effect of ASP (paired t test, ASP versus ASP + rolipram, p < 0.01). Enprofylline, OPC 3911, or rolipram had no significant effects on fractional FFA re-esterification per se (Fig. 6).

When the fractional FFA re-esterification values under basal conditions or with ASP (5.6 μM) or insulin (0.1 nM) in Fig. 1–3 were compared with those in Fig. 6, the values in Fig. 1–3 were slightly higher than in Fig. 6. The relative effects of ASP (5.6 μM) and insulin (0.1 nM), on the other hand, did not differ significantly between the experiments in Figs. 1–3 and 6 (ANOVA, p = not significant). The subjects in Fig. 1–3 did not differ significantly from those in Fig. 6 regarding age or BMI (ANOVA, p = not significant). No significant correlation was found between BMI or age on the one hand and basal, ASP- or insulin-induced fractional FFA re-esterification on the other hand. No significant gender difference was found in fractional FFA re-esterification values.

ASP and Insulin Effects on PDE Activity—To examine further the involvement of PDE in ASP and insulin action, PDE activity was measured in fat cells, following incubation with ASP or insulin (Fig. 7). These experiments were performed in an identical way on 7 subjects. It is shown that PDE3 activity was stimulated significantly in a concentration-dependent

### Table II

The effect of PDE blockers and norepinephrine on the action of ASP or insulin on FFA produced during lipolysis

|                | Basal (n = 10) | ASP (n = 9) | INS (n = 9) |
|----------------|---------------|-------------|-------------|
| Basal          | 100           | 73 ± 7      | 56 ± 7      |
| Rolipram       | 98 ± 7        | 76 ± 8      | 63 ± 13     |
| Enprofylline (n = 10) | 178 ± 33     | 148 ± 21    | 168 ± 33    |
| OPC 3911 (n = 9) | 208 ± 30     | 183 ± 37    | 205 ± 29    |
| NE (n = 8)     | 250 ± 31      | 188 ± 11    | 133 ± 17    |
| NE enprofylline (n = 8) | 289 ± 40    | 297 ± 39    | 279 ± 42    |

Fig. 6: The effect of the PDE inhibitors enprofylline (1 mM, n = 10), OPC 3911 (3 μM, n = 9), and rolipram (1 μM, n = 9) on fractional FFA re-esterification in the absence or presence of ASP or insulin. See legend to Figs. 3 and 4 for more details.
The release of FFA from human fat cells is a key feature in the regulation of the energy balance of the body. It is the net result of FFA formed during lipolysis and fractional FFA re-esterification. In the current study, several new mechanisms involved in the regulation of FFA release were observed. In particular, the involvement of ASP and the role of PDEs in FFA re-esterification were demonstrated.

Fat cell re-esterification can be measured with two different sensitive methods in vitro as follows: by simultaneous measurement of the release of glycerol and FFA using luminescence or by a dual radio isotope technique (42). We used the former method because it allows the utilization of very dilute fat cells suspensions. A number of lipolytic or antilipolytic substances are secreted by human fat cells, such as adenosine (43), lactate (33), prostaglandins (44), and tumor necrosis factor α (45). By using dilute fat cell suspensions, the possible influence of such substances on lipolysis is avoided or minimized. For example, we have shown, under the present incubation conditions, that the influence of adenosine release from human fat cells on lipolysis is negligible (46).

ASP, which stimulates triglyceride synthesis and glucose transport in human fat cells (16–17, 20–22), was found to markedly inhibit both basal and norepinephrine-stimulated FFA release from fat cells as well. This effect was due to a combination of the following two processes: ASP markedly increased the proportion of re-esterified FFA (fractional FFA re-esterification) and to a lesser extent it inhibited FFA produced during lipolysis. This pattern is different to that observed for insulin, which had marked effects on both lipolysis and fractional FFA re-esterification. When fat cells were incubated with maximally effective concentrations of each protein, ASP inhibited FFA produced during lipolysis to a lesser extent than insulin, whereas it stimulated fractional FFA re-esterification to the same or even greater extent than insulin.

We hypothesize that the effects of insulin and ASP on fractional FFA re-esterification are not secondary to the action of the two hormones on lipolysis. If stimulation of FFA re-esterification were secondary to inhibition of lipolysis, insulin and ASP would also affect the absolute rates of FFA esterification (i.e., primary esterification). However, no effects of insulin or ASP on primary esterification were observed. The insulin data confirm several earlier observations (9, 47–48). Only norepinephrine, which is lipolytic, was found to be effective in stimulating the rate of primary FFA esterification. The latter data confirm the results of previous studies with catecholamines (7, 48). On the other hand, the PDE blockers, enprofylline and OPC 3911, were also lipolytic in this study, but they did not affect the absolute rate of FFA re-esterification significantly (data not shown). Furthermore, the weak inhibitory effect of ASP on lipolysis could hardly explain its marked effect on fractional re-esterification.

Another novel observation is that ASP could markedly augment the effect of insulin on fractional FFA re-esterification, whereas it only marginally enhanced the antilipolytic effect. For example, insulin in combination with ASP reduced the lipolytic rate by 50–60% and inhibited the FFA release almost completely; the latter was probably due to a near maximal effect of the hormone combination on fractional FFA re-esterification. This suggests that the effects of ASP and insulin on FFA release are additive, which above all is due to a dual effect on fractional FFA re-esterification. The average ASP level in humans has been reported to be about 0.06 μM in fasting plasma increasing postprandially in fat tissue venous effluent to about 0.1 μM (49). In the present study, we observed significant in vitro effects of ASP at 0.1 and 0.5 μM, in the presence and absence of insulin, respectively. Thus, our findings may be of physiological relevance for circulating ASP, although some caution should be exercised in extrapolating from the in vitro to the in vivo situation. However, it is tempting to speculate that ASP and insulin act together in regulating FFA metabolism, increasing the proportion of re-esterified FFA so that FFA release is almost completely shut down in the postprandial state. Furthermore, increased release of FFA from fat cells in obesity and diabetes may not only relate to insulin resistance; a blunted response to ASP could have the same consequences.

The second aim of this study was to determine the intracellular signaling pathways that mediate the effects of ASP and insulin on fractional FFA re-esterification and lipolysis. From earlier studies it is known that ASP stimulates glucose transport and triglyceride synthesis through activation of a PKC-dependent pathway in human fibroblasts (19). Insulin, on the other hand, is known to exert its antilipolytic effect in human fat cells by phosphorylation of P13K which in turn activates PDE3 (8). In addition, P13K mediates the insulin effect on fractional re-esterification (10). The pathways distal to P13K that are involved in the effect of insulin on FFA re-esterification and in the action of ASP on FFA re-esterification and lipolysis have been unknown until now.

In this study, PKC blockers could not counteract the effects of ASP or insulin on fractional FFA re-esterification or lipolysis, suggesting that the actions of the two hormones on FFA release are not mediated by PKC. This is in contrast to earlier
The PDE3 blocker, mephalamine, counteracted, as expected, the effect of insulin on FFA release by reversing the effect of insulin on fractional FFA re-esterification and lipolysis. The effects of ASP, on the other hand, were not affected by mephalamine. So the pathway mediating the effect of ASP on FFA release does not involve PDE3. This strongly supports the hypothesis that ASP and insulin follow different pathways in their action on FFA release. The fact that the ASP receptor protein(s) has not been identified makes further research in this area difficult at present.

We next addressed the question whether a more distal pathway could be common for the actions of insulin and ASP on FFA metabolism. The interest was focused on PDE, which previously was found to mediate not only the antilipolytic effect of insulin but also the stimulatory effect of hormone on glucose transport in human fat cells (50). By catalyzing the hydrolysis of cAMP and cGMP, PDEs regulate intracellular concentrations and biological responses of these second messengers (8). PDEs constitute a group of structurally related enzymes that belong to at least nine related gene families (PDE 1–9), which differ in their primary structures, affinities for cAMP and cGMP, responses to specific effectors, and sensitivity for inhibitors and regulatory mechanisms (8, 51). Two PDEs (PDE3 and PDE4) have high affinity for cAMP and are present in fat cells (25). PDE3 can be distinguished from PDE4 by its high affinity for both cAMP and cGMP. Activation of PDE3 but not PDE4 plays a role in the antilipolytic effect of insulin in vivo in human fat tissue (33, 52). In this study, enprofylline, which is a non-selective PDE inhibitor that does not interact with adenylate cyclase, counteracted the actions of both ASP and insulin on FFA release by reversing their effects on fractional FFA re-esterification and lipolysis. The selective PDE3 blocker, OPC 3911, could also counteract these effects of ASP and insulin. The selective PDE4 blocker, rolipram, was not able to counteract the effect of insulin on fractional FFA re-esterification and lipolysis. Rolipram did not alter the effect of ASP on lipolysis either, but it counteracted slightly the effect of ASP on fractional FFA re-esterification. These results were in agreement with the results obtained with direct measurements of PDE activity. PDE3 activity in fat cells was stimulated with both ASP and insulin in a concentration-dependent manner, whereas PDE4 activity was only slightly stimulated with ASP. Thus, the effects of both ASP and insulin on FFA release appear to be mediated by PDE3. PDE4 seems to be involved in the action of ASP as well, although to a lesser extent than PDE3.

PDE blockade, using the PDE inhibitors enprofylline and OPC 3911, stimulated basal FFA production during lipolysis. It is likely that when lipolysis is increased, more ASP or insulin is required to reverse this process. One could say that the PDE inhibitors reversed the antilipolytic effect of ASP and insulin through their own lipolytic effect and not by specifically blocking PDE. This, however, seems unlikely. In the present study, stimulation of lipolysis with norepinephrine to a greater extent than observed with enprofylline did not prevent the inhibition of lipolysis by ASP or insulin. In contrast, enprofylline did not further increase lipolysis induced by norepinephrine alone, but ASP or insulin could not reverse lipolysis induced by norepinephrine combined with enprofylline. Norepinephrine does not maximally activate lipolysis in the present incubation conditions when compared with the selective β-adrenoreceptor agonist isoproterenol (53), since it is a β- and also an α₂-adrenoreceptor agonist. Thus, the data strongly indicate that the antilipolytic effect of ASP is mediated by PDE. The effects of the PDE blockers on insulin- and ASP-stimulated fractional FFA re-esterification cannot be explained by interactions with lipolysis. Although enprofylline and OPC 3911 stimulated lipolysis, they had no effect on their own on fractional FFA re-esterification.

The source of ASP was purified plasma. It is excluded that insulin contaminations were present in the ASP preparations. First, the ASP preparation was pure as evidenced by ion spray mass spectrometry analysis. Second, we could not detect insulin in the ASP preparations. Third, the mechanism of action of the two hormones differed considerably.

In this study, it was for practical reasons not possible to select the subjects for age, gender, and BMI. However, there was no apparent effect of these factors on the actions of ASP and insulin on fractional FFA re-esterification and lipolysis. Only subcutaneous fat cells were used, so whether there are regional differences in the effect of ASP on lipolysis and re-esterification remains to be investigated. There are some differences in insulin action on lipolysis in omental and subcutaneous fat cells (10).

In summary, the current study shows that ASP and insulin may play a major role in regulating basal- and catecholamine-stimulated FFA release from fat cells, through stimulatory effects on fractional primary FFA re-esterification and inhibitory effects on FFA produced during lipolysis. Insulin has marked effects on both processes, whereas ASP mainly stimulates fractional FFA re-esterification. The effects are mediated in both cases by PDE3. Some of the effect of ASP on fractional FFA re-esterification could also be mediated by PDE4. This suggests not only an important role of PDE in fat cell lipolysis but also in FFA re-esterification. The FFA re-esterification signaling pathway preceding PDE for insulin involves PI3K, but is not known for ASP yet, although PKC or PI3K do not seem to be involved.

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