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PAPER

Acylation stimulating protein stimulates insulin secretion

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Acylation stimulating protein (ASP) is a hormone produced by adipocytes and is of importance for the storage of energy as fat. We examined whether ASP might also have effects on islet function. In clonal INS-1 cells, ASP dose-dependently augmented glucose-stimulated insulin secretion. The lowest effective dose of ASP at 10 mmol/l glucose was 5 µmol/l. The effect was glucose-dependent because ASP did not increase insulin secretion at 1 mmol/l glucose but had clear effect at 10 and 20 mmol/l glucose. Similarly, ASP augmented glyceraldehyde-induced insulin secretion but the hormone did not enhance insulin secretion in response to depolarization by 20 mmol/l of KCl. ASP-induced insulin secretion was completely abolished by competitive inhibition of glucose phosphorylation by glucokinase with 5-thio-glucose and was partially inhibited by the calcium channel blocker, nifedipine, and by the protein kinase C inhibitor, GF109203. Furthermore, thapsigargin, an inhibitor of Ca²⁺-ATPase in the endoplasmic reticulum, did not affect ASP-induced insulin secretion. ASP (> 5 µmol/l) also augmented glucose-stimulated insulin secretion from islets isolated from C57BL/6J mice, and intravenous administration of ASP (50 nmol/kg) augmented the acute (1 and 5 min) insulin response to intravenous glucose (1 g/kg) in C57BL/6J mice. This was accompanied by an increased rate of glucose disposal. Minimal model analyses of data derived from the intravenous glucose tolerance test revealed that whereas ASP augmented insulin secretion, the hormone did not affect insulin sensitivity (S_i) or glucose effectiveness (S_G). We conclude that ASP augments glucose-stimulated insulin secretion through a direct action on the islet beta cells. The effect is dependent on glucose phosphorylation, calcium uptake and protein kinase C. Stimulation of insulin secretion by ASP *in vivo* results in augmented glucose disposal.

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Keywords: acylation stimulating protein; ASP; insulin secretion; insulin sensitivity; INS cells; *in vivo*; mice

Introduction

During recent years, it has become evident that adipocytes express and secrete a number of hormones involved in the regulation of energy and substrate metabolism including leptin, adiponectin, resistin and acylation stimulating protein (ASP).^{1–3} ASP is a 8.9 kDa hormone produced in the adipocytes through an interaction of complement factor C3 with factor B and factor D (also called adipsin), which results in the formation of C3a-des-Arg, also called ASP.⁴ ASP is released into the circulation and has been shown to augment triglyceride synthesis and storage in adipocytes in combination with stimulation of glucose uptake and diacylglycerol

acyltransferase and inhibition of hormone-sensitive lipase via phosphodiesterase.^{5–7} This would suggest that ASP contributes to the storage of energy as lipids. Such a function is supported by data indicating that ASP administration increases triglyceride clearance from plasma,⁸ and that ASP-deficient (C3–/–) mice exhibit delayed postprandial lipid clearance, reduced adipose tissue depots and are resistant to obesity induced by high-fat diet.^{9,10} Involvement by ASP in the regulation of energy storage is also supported by reports that plasma levels of ASP are increased in obesity¹¹ and reduced in fasting¹² and in postobese women.¹³ Adipose tissue release of ASP increases postprandially, with a peak release observed at 4–5 h after meal ingestion, although circulating levels remain constant.¹⁴

Whether ASP is involved in the regulation of glucose homeostasis is not known. ASP-deficient mice exhibit reduced fasting insulin levels and improved glucose tolerance.⁹ This would suggest that ASP reduces insulin

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sensitivity. This idea is supported by a study in humans showing that plasma ASP correlates inversely to glucose disposal during a euglycemic clamp,¹⁵ although this finding was not reproduced in a subsequent study.¹⁶ An effect of ASP to reduce insulin sensitivity could be explained by its action to increase triglyceride storage, because intracellular lipid accumulation in some tissues including liver and muscle is associated with reduced insulin sensitivity.^{17,18} In the present study, we have examined the direct effect of ASP on insulin secretion *in vitro* in insulin-producing clonal INS-1 cells and in isolated mouse islets. In addition, the effects of ASP on insulin secretion were examined *in vivo* in C57BL/6J mice along with the influences of ASP on insulin sensitivity and on glucose disposal.

Methods

Studies in INS-1 cells

INS-1 cells (kindly provided by Dr C Wollheim, Geneva, Switzerland) were cultured in plastic flasks at 37°C in 5% CO₂/95% air in RPMI medium supplemented with 50 µmol/l mercaptoethanol (GIBCO BRL, Paisley, UK), 2.06 mmol/l L-glutamine (Life Technologies, Täby, Sweden), 10% fetal bovine serum, 100 U/ml penicillin and 2.5 µg/ml amphotericin B (all from Kebo Laboratory, Spånga, Sweden). The cells were divided after 7 days (about 75% confluence), washed twice in a HEPES medium containing (in mmol/l) 125 NaCl, 5.9 KCl, 1.28 CaCl₂, 1.2 MgCl₂, 25 Hepes and 0.1% bovine serum albumin (pH 7.36). In studies on insulin secretion, cells were preincubated for 30 min at 37°C in 200 µl of the medium supplemented with 1 mmol/l glucose. Thereafter, the cells were incubated for 60 min in the medium supplemented with glucose, glyceraldehyde, nifedipine, GF109203, thapsigargin with or without addition of ASP according to the protocols. Following the incubation, 150 µl of the medium were removed and centrifuged at 350 × g for 5 min, whereafter aliquots of 50 µl were saved at -20°C until analysis. GF109203 was purchased from Biomol. Res. Labs., Plymouth Meeting, PA, USA. ASP was prepared as described previously¹⁹ and its purity (99%) was ascertained by ion spray mass spectrophotometry.²⁰ Other substances and reagents were from Sigma Chemical Co., St Louis, MO, USA.

Studies in mice

Female C57BL/6J mice, weighing 23.0 ± 0.3 g, obtained from the Taconic M&B A/S, Ry, Denmark, were used for this study. The mice were fed a normal laboratory chow diet and tap water *ad libitum*. The study was approved by the Ethics Committee of Lund University.

Islet studies

Islets were isolated by collagenase digestion, hand-picked under a stereomicroscope and incubated overnight in RPMI 1640 medium supplemented with 10% fetal calf serum,

100 U/ml penicillin G, 0.1 mg/ml streptomycin and 2.5 µg/ml amphotericin B. Islets were then incubated in groups of three in 96-well microtiter plates in a Hepes buffer containing 3.3, 11.1 or 22.2 mmol/l D-glucose with or without 10 µmol/l ASP. After 60 min incubation at 37°C, the supernatant was collected and stored at -20°C until analyzed for insulin concentration.

In vivo study

The animals were anesthetized with an intraperitoneal injection of midazolam (0.14 mg/mouse; Dormicum[®], Hoffman-La-Roche, Basel, Switzerland) and a combination of fluanison (0.28 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm[®], Janssen, Beerse, Belgium). After 30 min, a blood sample (75 µl) was taken from the retrobulbar, intraorbital, capillary plexus in a 100 µl pipette that had been prerinsed in heparin solution (100 U/ml in 0.9% NaCl; Lövens, Ballerud, Denmark). Thereafter, D-glucose (British Drug Houses, Poole, UK) was injected intravenously over 3 s at the dose of 1 g/kg in a tail vein without flushing of the 27 gauge needle after injection, either alone or together with ASP (50 nmol/kg body weight; resulting in a peak level of approximately 5–10 times the circulating level). The injection volume load was 10 µl/g body weight. Additional blood samples (75 µl each) were collected at 1, 5, 10, 20, 30 and 50 min. Plasma samples were immediately separated and stored at -20°C until analyzed for insulin and glucose concentrations.

Analyses

Insulin concentration was determined by a double-antibody radioimmunoassay using guinea pig anti rat insulin antibodies, ¹²⁵I-labeled human insulin and, as standard, rat insulin (Linco Res., St Charles, MO, USA). Glucose was measured by the glucose oxidase technique.

Statistics and calculations

Data and results are reported as means ± s.e.m. Statistical comparisons between the groups were performed with unpaired Student's *t*-test. ANOVA was used for multiple comparisons. Insulin and glucose data from the intravenous glucose tolerance test (IVGTT) were analyzed with the minimal model technique as previously described.²¹ This analysis provides parameter *S*₁ (insulin sensitivity index), which is defined as the ability of insulin to enhance net glucose disappearance and inhibit glucose production, and the parameter *S*_G, which is the glucose effectiveness, representing net glucose disappearance *per se* from plasma without any change in dynamic insulin. Acute insulin response (AIR; mean of 1 and 5 min insulin levels after subtraction of baseline value) and the area under the 50 min curve of insulin concentration (ie the total insulin response, AUC_{insulin}; calculated by the trapezoid rule) were determined. The glucose tolerance index, *K*_G, was obtained as the

slope of the logarithmic transformation of the individual plasma glucose values in the interval 1–20 min after glucose injection.

Results

Effects of ASP on glucose-stimulated insulin secretion in INS-1 cells

Glucose-stimulated insulin secretion from INS-1 cells was augmented by adding ASP to the incubation media. The effect of ASP was dose-dependent with 5 $\mu\text{mol/l}$ being the lowest effective dose (Figure 1, upper panel). The effect also required a threshold concentration of glucose because ASP did not increase insulin secretion in the presence of 1 mmol/l glucose but augmented insulin secretion in the presence of 10 or 20 mmol/l glucose (Figure 1, lower panel). ASP also augmented the insulin response to glyceraldehyde at 10 and 20 mmol/l, but not at 1 mmol/l (Figure 2, upper panel). In contrast, ASP did not increase insulin secretion elicited by depolarization by a high concentration of K^+ (Figure 2, lower panel).

To examine whether the insulinotropic action of ASP is dependent on glucose phosphorylation, cells were incubated in the presence of 5-thio-glucose (10 mmol/l), which is a potent competitive inhibitor of glucokinase.^{22,23} When the cells were incubated in the presence of 10 mmol/l 5-thio-glucose, ASP-stimulated insulin secretion was completely abolished (Figure 3). Furthermore, ASP-induced insulin secretion was inhibited by approximately 50% by nifedipine (5 $\mu\text{mol/l}$), which is a blocker of the L-type Ca^{2+} channels.²⁴ Thus, in the absence of nifedipine, ASP augmented glucose-stimulated insulin secretion by $7.4 \pm 0.8 \text{ nmol/l}$ and this was reduced by nifedipine to $4.0 \pm 0.4 \text{ nmol/l}$ ($P < 0.001$; Figure 3). Activation of protein kinase C (PKC) is known to contribute to glucose-stimulated insulin secretion.^{25,26} We found that bisindolylmaleimide (GF109203; 2 $\mu\text{mol/l}$), which inhibits PKC,²⁷ inhibited ASP-induced insulin secretion by approximately 30%. Thus, whereas ASP in the absence of GF109203 augmented glucose-stimulated insulin secretion by $7.5 \pm 0.9 \text{ nmol/l}$, the inhibitor reduced the effect of ASP to $4.8 \pm 0.5 \text{ nmol/l}$ ($P < 0.001$). Finally, by combining nifedipine and GF109203 together, ASP-induced insulin secretion was completely abolished (Figure 3). In contrast, intracellular Ca^{2+} stores do not appear to be required for ASP-induced insulin secretion, because incubation of the cells with thapsigargin, which inhibits the $\text{Ca}^{2+}/\text{ATPase}$ in the endoplasmic reticulum thereby depleting the intracellular Ca^{2+} stores,²⁸ did not affect ASP-induced insulin secretion (Figure 3).

Effects of ASP on glucose-stimulated insulin secretion in isolated mouse islets

To examine whether ASP also affects glucose-stimulated insulin secretion in normal islets, isolated islets from C57BL/6J mice were incubated in the presence of ASP at

Figure 1 Medium insulin concentration after 60 min incubation of INS-1 cells in the presence of 1 or 10 mmol/l glucose with or without addition of ASP at different concentrations (upper panel) or in the presence of glucose at 1, 10 or 20 mmol/l with or without ASP at 10 $\mu\text{mol/l}$ (lower panel). Means $\pm$ s.e.m. are shown. In the upper panel asterisks indicate probability level of random difference vs 10 mmol/l glucose without ASP (ANOVA with Bonferroni *post hoc* analysis) and in the lower panel asterisks indicate probability level of random difference between groups (Student's unpaired *t*-test). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There were 16 incubations in each column.

different glucose concentrations. ASP potentiated glucose-stimulated insulin secretion (Figure 4). The effect showed similar characteristics to that observed in INS-1 cells, that is, being glucose-dependent and requiring a threshold level of glucose.

Effects of ASP on glucose-stimulated insulin secretion in mice

To determine whether ASP affects glucose-stimulated insulin secretion *in vivo*, anesthetized mice were injected intra-

Figure 2 Medium insulin concentration after 60 min incubation of INS-1 cells in the presence of 1, 10 or 20 mmol/l glyceraldehyde with or without addition of ASP at 10 µmol/l (upper panel) or in the presence of 1 or 10 mmol/l glucose with or without ASP at 5.9 or 20 mmol/l of KCl (lower panel). Means $\pm$ s.e.m. are shown. Asterisks indicate probability level of random difference between groups (Student's *t*-test for unpaired conditions). ** $P < 0.01$. There were 18–24 incubations in each column.

venously with glucose alone or in combination with ASP (50 nmol/kg). ASP augmented the insulin response to glucose (Figure 5). The effect was rapid in onset and short-lived and evident during the first 5 min after injection, when the AIR to glucose was increased from 346 ± 46 pmol/l in controls to 571 ± 95 pmol/l by ASP ($P = 0.042$). This resulted in an increased glucose disposal rate. K_G was augmented by ASP from $2.4 \pm 0.2\%/min$ in controls to $2.9 \pm 0.2\%/min$ ($P = 0.033$; Table 1). After the initial 5 min exaggeration of the insulin response to glucose, plasma insulin levels were slightly lower in the animals that received ASP in combination with glucose at 20 and 30 min ($P < 0.05$). The total AUC_{insulin} throughout the 50 min study period was not significantly different between the groups. Minimal model

Figure 3 Medium insulin concentration after 60 min incubation of INS-1 cells in the presence of 1 or 10 mmol/l glucose. Incubations at 10 mmol/l glucose were performed with (black bars) or without (open bars) addition of ASP at 10 µmol/l. Each panel shows incubations under control conditions (no inhibitor added) or incubations in the presence of one inhibitor: the inhibitor of glucokinase, 5-thio-glucose (10 mmol/l; upper left panel), the Ca^{2+} channel blocker, nifedipine (5 µmol/l; upper right panel), the inhibitor of PKC, bisindolylmaleimide (GF109203; 2 µmol/l; middle left panel), the combination of nifedipine and GF109203 (middle right panel) or the inhibitor of Ca^{2+} /ATPase, thapsigargin (lower left panel). Means $\pm$ s.e.m. are shown. Asterisks indicate significance between conditions with vs without ASP, that is, conditions without and with inhibitor analyzed separately (Student's *t*-test for unpaired conditions). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There were 16–24 incubations in each bar.

analysis of the glucose and insulin data showed that ASP did not affect insulin sensitivity (S_I) or glucose effectiveness (S_G ; Table 1). A significant linear correlation was found between AIR and K_G ($r = 0.52$, $P = 0.010$).

Discussion

Previous studies have suggested that the adipocyte-derived hormone ASP promotes energy storage as lipids through a direct action on adipocytes.^{3–10} This study shows that ASP may be involved also in the regulation of glucose homeostasis and islet function because the hormone augments glucose-stimulated insulin secretion both *in vitro* and *in vivo*,

Figure 4 Medium insulin concentration after 60 min incubation of isolated mouse islets in the presence of varying concentrations of glucose with or without ASP at 10 µmol/l as indicated. Means $\pm$ s.e.m. are shown. Asterisks indicate probability level of random difference between conditions with vs without ASP (Student's *t*-test for unpaired conditions). ** $P < 0.01$, *** $P < 0.001$. There were 24 incubations in each column.

and that this action results in augmentation of glucose disposal rate under *in vivo* conditions.

In the clonal INS-1 cells, ASP dose-dependently augmented insulin secretion in a glucose-dependent manner. This suggests that ASP augments insulin secretion by promoting the efficiency of the glucose signaling in the beta cells. In insulin-producing cells, glucose is taken up and phosphorylated. These processes are mediated by the glucose transporter, GLUT-2, and a high K_m hexokinase, type IV (glucokinase). Glucokinase is of particular importance, being the 'glucose sensor' and the rate-limiting step in glucose-stimulated insulin secretion.²⁹ As a tool to examine whether this process is required also for the ASP-induced potentiation of glucose-stimulated secretion, we used 5-thio-glucose, which is known to inhibit glucokinase in beta cells.²³ We found that 5-thio-glucose abolished ASP-induced insulin secretion. Since glyceraldehyde-induced insulin secretion was also potentiated by ASP, our results imply that ASP augments insulin secretion by generating a signal downstream of glyceraldehyde-3-phosphate.

Our results further implicate two separate mechanisms in the effects of ASP to stimulate insulin secretion. A first step is the uptake of Ca^{2+} through opening of L-type Ca^{2+} channels, because nifedipine, which blocks these Ca^{2+} channels in beta cells,²⁴ inhibited ASP-induced insulin secretion by approximately 50%. The Ca^{2+} channels are normally opened by depolarization instituted by ATP-induced closure of K_{ATP} channels.²⁵ ASP may thus augment insulin secretion through opening of Ca^{2+} channels by increasing the generation of ATP. However, also in the presence of nifedipine, a considerable fraction ($\approx 50\%$) of the ASP-induced insulin secretion remained intact. We found that this remaining ASP-induced insulin secretion was inhibited

Figure 5 Plasma glucose and insulin levels before and after the i.v. injection of glucose (1 g/kg) alone or together with ASP (50 nmol/kg) in anesthetized mice. Means $\pm$ s.e.m. are shown. Asterisks indicate the probability level of random difference between the groups (Student's *t*-test for unpaired conditions). * $P < 0.05$, ** $P < 0.01$. There were 12 animals in each group.

by GF109203, which is an inhibitor of classical PKC subtypes.²⁷ PKC is involved in insulin secretion by first being activated by glucose through diacylglycerol formed after phosphoinositide hydrolysis instituted by phospholipase C, and then translocated to the cell membrane and activating the exocytosis of insulin.²⁶ The results thus suggest that ASP stimulates insulin secretion by augmenting the action of glucose to open Ca^{2+} and to activate PKC. Interestingly, these effects resemble the actions of ASP in adipocytes, where the hormone has been shown to enhance glucose uptake through translocation of glucose transporters by a mechanism involving translocation and activation of PKC.^{4,30-32} In contrast, release of Ca^{2+} from intracellular stores does not seem to be a mechanism of ASP in beta cells, because depletion of Ca^{2+} stores with thapsigargin²⁸ did not affect ASP-induced insulin secretion. The involvement of proximal mechanisms for ASP-induced insulin secretion is also supported by the results that the hormone was unable to

Table 1 Parameters from the minimal model analysis of insulin and glucose data during the IVGTT in mice injected with glucose alone (1 g/kg; $n=12$) or glucose together with ASP (50 nmol/kg; $n=12$)

Parameter (unit)	Glucose alone	Glucose+ASP	P-value
AIR (pmol/l)	346 ± 46	571 ± 95	0.042
AUC _{insulin} (nmol/l × 50 min)	13.3 ± 1.1	11.9 ± 0.9	0.26
K_G (%/min)	2.4 ± 0.2	2.9 ± 0.2	0.033
S_I (10 ⁻⁴ /min)/(pmol/l)	1.12 ± 0.21	1.41 ± 0.18	0.32
S_G (/min)	0.059 ± 0.007	0.072 ± 0.006	0.16

Means ± s.e.m. are shown. *P*-value indicates the probability level of random difference between the two groups. AIR = acute (1–5 min) insulin response to i.v. glucose, AUC_{insulin} = incremental area under the insulin curve during 50 min after i.v. glucose, K_G = glucose elimination rate during 1–20 min after i.v. glucose, S_I = insulin sensitivity index and S_G = glucose effectiveness; the last two parameters are derived from the minimal model analyses of data.

augment insulin secretion stimulated by high K^+ . Stimulation by K^+ bypasses the proximal effects of glucose and increases an uptake of Ca^{2+} through a direct depolarization effect, which is a K_{ATP} channel-independent pathway.^{25,33}

We also found that ASP augments insulin secretion *in vivo* in mice. Intravenous administration of ASP in combination with glucose during an IVGTT potentiated glucose-stimulated insulin secretion. The effect was rapid in onset and observed within 1 min after administration. This augmented glucose-stimulated insulin secretion resulted in increased rate of glucose disposal. This induced slightly lower glucose levels at the later time points during the IVGTT, and is likely to explain the reduced plasma insulin levels at these later time points. The use of the minimal model analysis of the data obtained from the IVGTT also allowed us to investigate the potential influence of ASP on insulin sensitivity and glucose effectiveness, that is, the glucose disappearance independent of any dynamic change of insulin.²¹ The analysis revealed, however, that these parameters were not affected by ASP. Therefore, the augmented glucose disposal seen after administration of ASP appears to be mediated by the increased insulin secretion. Our finding that ASP did not affect insulin sensitivity might seem to contradict earlier observations that plasma ASP levels in humans correlate inversely to glucose disposal during a euglycemic clamp¹⁵ and that ASP-deficient mice exhibit reduced plasma insulin levels as a sign of increased insulin sensitivity.⁹ However, it has to be emphasized that during chronic studies the action of ASP to alter triglyceride stores and adipose tissue mass might indirectly influence insulin sensitivity, an action which may be distinct from direct actions observed after short-term administration of the hormone.

In summary, the results presented here provide evidence that, in addition to its involvement in the local regulation of lipid formation in adipocytes,^{3–10} ASP may also have a role in the regulation of islet function by augmenting glucose-stimulated insulin secretion via a direct action on the beta cells. This highlights an interaction between the adipocytes and the regulation of islet function.³⁴ Furthermore, in

addition to promoting glucose uptake and utilization, insulin also prominently inhibits lipolysis in adipocytes.³⁵ Therefore, based on the available data, the main actions of ASP appear to be its effects to promote energy storage as lipids, both directly to stimulate lipid synthesis in adipocytes,^{5–7} and indirectly by promoting insulin secretion and thereby inhibiting lipolysis. Further studies will be required to examine whether perturbations of this regulation may be involved in metabolic disorders, such as diabetes and obesity.

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