

# Altered Insulin Secretion Associated With Reduced Lipolytic Efficiency in $aP2^{-/-}$ Mice

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Recent studies have shown that genetic deficiency of the adipocyte fatty acid-binding protein (aP2) results in minor alterations of plasma lipids and adipocyte development but provides significant protection from dietary obesity-induced hyperinsulinemia and insulin resistance. To identify potential mechanisms responsible for this phenotype, we examined lipolysis and insulin secretion in  $aP2^{-/-}$  mice.  $\beta$ -Adrenergic stimulation resulted in a blunted rise of blood glycerol levels in  $aP2^{-/-}$  compared with  $aP2^{+/+}$  mice, suggesting diminished lipolysis in  $aP2^{-/-}$  adipocytes. Confirming this, primary adipocytes isolated from  $aP2^{-/-}$  mice showed attenuated glycerol and free fatty acid (FFA) release in response to dibutyryl cAMP. The decreased lipolytic response seen in the  $aP2^{-/-}$  mice was not associated with altered expression levels of hormone-sensitive lipase or perilipin. The acute insulin secretory response to  $\beta$ -adrenergic stimulation was also profoundly suppressed in  $aP2^{-/-}$  mice despite comparable total concentrations and only minor changes in the composition of systemic FFAs. To address whether levels of specific fatty acids are different in  $aP2^{-/-}$  mice, the plasma FFA profile after  $\beta$ -adrenergic stimulation was determined. Significant reduction in both stearic and *cis*-11-eicosenic acids and an increase in palmitoleic acid were observed. The response of  $aP2^{-/-}$  mice to other insulin secretagogues such as arginine and glyburide was similar to that of  $aP2^{+/+}$  mice, arguing against generally impaired function of pancreatic  $\beta$ -cells. Finally, no aP2 expression was detected in isolated pancreatic islet cells. These results provide support for the existence of an adipo-pancreatic axis, the proper action of which relies on the presence of aP2. Consequently, aP2's role in the pathogenesis of type 2 diabetes might involve regulation of both hyperinsulinemia and insulin resistance through its impact on both lipolysis and insulin secretion. *Diabetes* 48:1987–1994, 1999

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aP2, adipocyte fatty acid-binding protein;  $\beta$ -AR,  $\beta$ -adrenergic receptor; FABP, fatty acid-binding protein; FFA, free fatty acid; HSL, hormone-sensitive lipase; mal1, keratinocyte fatty acid-binding protein; PCR, polymerase chain reaction.

The adipocyte fatty acid-binding protein (aP2) is a member of the family of intracellular fatty acid-binding proteins (FABPs) (1,2) expressed at high levels in the cytosol of brown and white adipocytes (3). Like most FABPs, aP2 can bind a single fatty acid molecule, with similar affinity for long-chain saturated and unsaturated fatty acids (free fatty acids [FFAs]) (4). Although its protein structure (5–8), ligand binding (9), and ligand transfer properties (10–13) have been thoroughly studied in cell-free systems, understanding of the biological functions of aP2 has remained incomplete at the cellular level and in vivo. Proposed functions of aP2 and other FABPs include the shuttling of FFAs through the aqueous cytosol and buffering to prevent adverse detergent effects of FFAs on lipid membranes.

We recently generated aP2-deficient ( $aP2^{-/-}$ ) mice by targeted disruption of the *aP2* gene (14). Although aP2 is the predominant cytosolic fatty acid-binding activity in white adipocytes,  $aP2^{-/-}$  mice are healthy, develop apparently normal adipose tissue, and exhibit only minor alterations in their steady-state lipid metabolism under standard laboratory conditions. This lack of a significant effect of aP2 deficiency was potentially the consequence of the compensatory upregulation at the mRNA level of keratinocyte fatty acid-binding protein (mal1) (15), an otherwise minor adipocyte FABP, in adipose tissue of  $aP2^{-/-}$  mice. In contrast to the mild phenotype of the lean mice under standard laboratory conditions, a striking metabolic consequence of the aP2 deficiency was noticed when  $aP2^{-/-}$  animals were placed on a high-fat diet to induce obesity (14). The obese  $aP2^{-/-}$  mice were significantly protected from hyperinsulinemia and insulin resistance compared with their wild-type ( $aP2^{+/+}$ ) littermates. This protection from dietary obesity-induced insulin resistance in the absence of aP2 suggests that adipocyte fatty acid metabolism is a critical component of the mechanisms leading to systemic insulin resistance in obesity.

Among potentially relevant parameters of adipocyte fatty acid metabolism, the lipolytic breakdown of stored triglycerides is of particular interest. Major physiologic regulation of this metabolic event occurs through activation of  $\beta$ 1- and  $\beta$ 3-adrenergic receptors ( $\beta$ -ARs) located on the surface of adipocytes (16–18). At the intracellular level, lipolysis is a highly regulated process, the control of which is mediated through the key enzyme, hormone-sensitive lipase (HSL) (19,20), and potentially through perilipin, a protein located at the surface of the lipid droplet (21,22). Lipolysis and FFA release from adipose tissue are commonly increased in obesity. This increase has been suggested to be an important

factor in the initiation of hyperinsulinemia and insulin resistance (23). In support of this hypothesis, experimental alterations in FFA metabolism have been demonstrated to influence both insulin secretion and peripheral insulin action (24–27). The release of fatty acids from adipocytes could involve FABP activity at several stages, including the efficiency of flux from the lipid droplet to the plasma membrane or intermediary metabolic activities. Hence, we examined the effects of *aP2* deficiency on adipocyte lipolysis and the subsequent acute insulin secretory response. Our results demonstrate that *aP2* plays an important role in regulation of both these parameters.

## RESEARCH DESIGN AND METHODS

**Animals and experimental conditions.** Mice deficient for *aP2* were backcrossed 12 generations to C57Bl6/J mice to create congenic *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice. Mice were kept on a 7:00 A.M./7:00 P.M. light/dark schedule and given a standard diet. Unless otherwise indicated, studies were performed with 9- to 10-week-old male mice, and experiments and sample collection took place in the afternoon after food withdrawal for 6 h. Postprandial experiments were conducted at 11:00 P.M. after withdrawing food during the daylight period and refeeding at 7:00 P.M. Isoproterenol (Sigma, St. Louis, MO) and CL 316,243 (provided by Dr. Kurt Steiner, Wyeth Ayerst Research, Princeton, NJ) were dissolved in phosphate-buffered saline and injected intraperitoneally at a dose of 10 mg/kg (isoproterenol) and 0.1 mg/kg (CL 316,243). Blood was collected from the orbital plexus after anesthetizing animals with methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL). Serum was frozen in aliquots and stored at -20°C. Enzymatic assays were used for the determination of serum FFAs (Wako, Richmond, VA) and glycerol (Sigma). Serum insulin, C-peptide, and leptin levels were measured using radioimmunoassays from Linco Research (St. Charles, MO). Glucose levels were determined using blood glucose strips (MediSense, Waltham, MA).

**Preparation of primary adipocytes and lipolysis experiments.** Epididymal white adipose tissue was obtained from female *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (24 weeks old) that had been maintained on standard rodent food. Primary adipocytes were isolated from at least two mice per experiment using a standard collagenase technique (28,29) modified by Meyers et al. (30). The lipolysis assays were conducted essentially as described (30). Each assay point was performed in triplicate using 30 mg wet weight of cells. After incubation, aliquots of medium were separated from cells and used to assay glycerol and FFAs using commercial assay reagents.

**Quantitation of *aP2* and mall in primary adipocytes.** Protein for determination of FABPs in isolated adipocytes was harvested by homogenizing cells in 50 mmol/l HEPES (pH 7.2), 150 mmol/l NaCl, 5 mmol/l EDTA, 10 μmol/l leupeptin, and 0.2 mg/ml Pefabloc (2 ml/g wet weight) at 20°C. The 2000g infranantant (20°C) of the homogenate was spun at 100,000g and 4°C. The resulting supernatant (cytosol) was assayed for FABPs using an immunoblot method described previously (31) and antisera raised against full-length human mall and *aP2*. Proteins were quantitated by standardization to recombinant murine *aP2* and mall. Mall in extracts from *aP2*<sup>+/+</sup> cells might be overestimated due to cross-reactivity (5–10%) of the mall antibody to murine *aP2*.

**RNA extraction and Northern blot analysis.** Epididymal white fat pads were excised, frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted by a CsCl gradient protocol (32), and 15 μg RNA per lane was used for Northern blot analysis as described (33). The cDNA clones used for the generation of labeled probes (HSL, perilipin) were provided by Dr. C. Londos, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. A human ribosomal protein probe (36B4) (34) was used as loading control.

**Reverse transcription-polymerase chain reaction.** cDNA prepared from mouse islets (SV129 × C57Bl/6) was provided by Dr. Markus Stoffel, Rockefeller University. Control cDNAs were prepared from total RNA of mouse tissues (C57Bl/6) using a commercially available kit (Invitrogen, Carlsbad, CA). Primers to detect *aP2* were 5'-CAGAAGTGGGATGGAAAGTCG-3' (forward) and 5'-CGACT-GACTATTGTAGTGTGTTGA-3' (reverse). Amplification was performed with *Taq* polymerase at 55°C annealing temperature using a standard polymerase chain reaction (PCR) buffer (Gibco BRL, Gaithersburg, MD) containing 2 mmol/l MgCl<sub>2</sub>. PCR primers and conditions for amplification of HNF4α have been described (35).

**Protein extraction and immunoblot analysis.** Protein was extracted from epididymal fat pads essentially as described (36), except that 0.2 mg/ml Pefabloc (Boehringer Mannheim, Mannheim, Germany) was used instead of phenylmethylsulfonyl fluoride. For immunoblot analysis of HSL, clear infranantant of the extracts was concentrated threefold using Microcon 30 columns (Amicon; Millipore, Bedford, MA). Protein (150 μg) was denatured, reduced, and separated on an SDS polyacrylamide (7.5%) gel. For analysis of perilipin, a lipid-associated protein, lipid-con-

taining supernatant corresponding to 20 mg of fat pad was supplemented with SDS (5%), reduced, denatured (10 min, 100°C), and separated on an SDS polyacrylamide (10%) gel. Proteins were transferred to a nitrocellulose membrane and detected using polyclonal anti-HSL or anti-perilipin sera (provided by Dr. C. Londos).

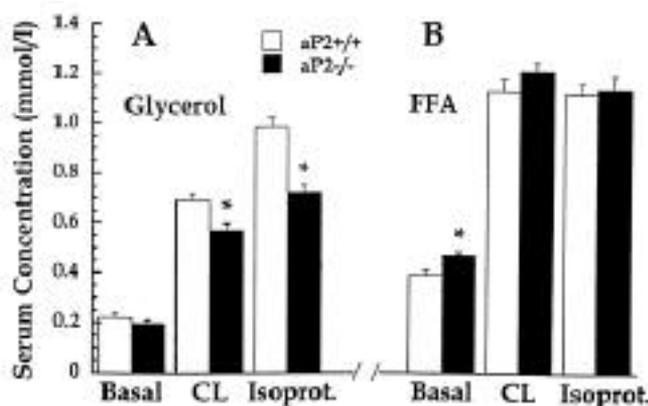
**Fatty acid analysis.** Total lipids were extracted from serum by the method of Dole and Meinertz (37). FFAs were separated from esterified fatty acids by thin-layer chromatography as described (38) using silica gel and a mixture of petroleum ether:diethyl ether:acetic acid (80:20:1) as the stationary and mobile phases, respectively. Fatty acids were methylated by incubation (50°C, 1 h) with methanol/acetyl chloride (20:1), evaporated, redissolved in iso-octane, and separated by gas chromatography. Individual fatty acids were identified by comparison with purified standards.

**Statistical analysis.** Calculations were performed using Microsoft Excel. The data are presented as means ± SE, and *P* values represent two-tailed Student's *t* tests.

## RESULTS

**Lipolysis studies in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice.** Lipolysis is an adipocyte function likely to be altered in *aP2*<sup>-/-</sup> mice and also a factor that might contribute to the protection from insulin resistance observed in these animals. To test this hypothesis in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice, we first examined lipolysis *in vivo* using intraperitoneal administration of CL 316,243 (0.1 mg/kg), a specific agonist of the β<sub>3</sub>-AR (39) that, in rodents, is predominantly expressed in adipose tissue (40–42). In a subset of experiments, we also used isoproterenol (10 mg/kg), a general β-adrenergic agonist. Circulating levels of FFAs and insulin have been shown to be maximally elevated between 10 and 15 min after intraperitoneal administration of CL 316,243 (43) (see below). In line with these data, blood was collected 15 min after injection in most of our experiments. FFAs and glycerol were determined as parameters of lipolysis.

First, lipolysis experiments were performed using animals in their daytime resting period. Basal glycerol levels in this physiologic state were similar in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (0.20 ± 0.014 vs. 0.22 ± 0.017 mmol/l). On injection of CL 316,243, glycerol levels rose in both groups. However, the extent of this rise was significantly less in the *aP2*<sup>-/-</sup> mice compared with *aP2*<sup>+/+</sup> animals (0.57 ± 0.027 vs. 0.70 ± 0.024 mmol/l; *P* < 0.005). An even more pronounced difference was evident after administration of isoproterenol (0.72 ± 0.031 vs. 0.98 ± 0.039 mmol/l; *P* < 0.001) (Fig. 1A), suggesting



**FIG. 1.** Basal and stimulated lipolysis in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice. **A:** Glycerol levels in serum collected from untreated animals (Basal; *n* = 17) and from animals 15 min after intraperitoneal administration of CL 316,243 (CL; *n* = 20) or isoproterenol (Isoprot.; *n* = 7). **B:** Serum FFA levels of untreated animals (*n* = 21) and 15 min after intraperitoneal administration of CL 316,243 (*n* = 20) or isoproterenol (*n* = 7). \*Significant differences between *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (*P* < 0.005).

TABLE 1  
Basal and  $\beta$ 3-AR-stimulated levels of metabolic parameters in the postprandial state

	<i>aP2</i> <sup>+/+</sup>		<i>aP2</i> <sup>-/-</sup>	
	Basal	Stimulated	Basal	Stimulated
FFAs (mmol/l)	0.31 ± 0.06	0.98 ± 0.09	0.26 ± 0.04	0.80 ± 0.08
Glycerol (mmol/l)	0.18 ± 0.01	0.42 ± 0.02	0.18 ± 0.03	0.35 ± 0.02*
Insulin (ng/ml)	3.2 ± 0.96	8.4 ± 2.4	2.4 ± 0.39	3.3 ± 0.9*
Glucose (mg/dl)	174 ± 6.0	148 ± 9.8	161 ± 8.2	169 ± 15.1
Leptin (ng/ml)	3.3 ± 0.1	2.9 ± 0.2	3.3 ± 0.3	2.6 ± 0.2

Blood for basal measurements was collected from the same set of animals 1 h before intraperitoneal administration of CL 316,243 and further collection of samples 15 min later (stimulated). \*Significant differences between *aP2*<sup>-/-</sup> ( $n = 8$ ) and *aP2*<sup>+/+</sup> ( $n = 6$ ) mice.  $P < 0.05$ .

reduced lipolysis in the *aP2*<sup>-/-</sup> mice. In agreement with our previous results (14), basal blood levels of FFAs were found to be moderately higher in *aP2*<sup>-/-</sup> compared with *aP2*<sup>+/+</sup> mice ( $0.47 \pm 0.014$  vs.  $0.39 \pm 0.019$  mmol/l;  $P < 0.005$ ). A significant elevation of FFA levels was observed after  $\beta$ -adrenergic stimulation in both groups. Blood levels of FFAs rose to equal levels in both genotypes after stimulation with either CL 316,243 or isoproterenol (Fig. 1B), although fold increase over basal was lower in the *aP2*<sup>-/-</sup> mice.

In the postprandial state, lipolysis is suppressed because of the negative action of insulin (44,45). We next tested whether an attenuation of lipolysis could also be detected in the *aP2*<sup>-/-</sup> mice under this physiologic condition by performing experiments 4 h after the onset of the dark period and availability of food. As expected, basal FFA and glycerol levels in the postprandial state were decreased compared with resting animals, consistent with the observed elevated basal levels of insulin. The basal levels of both glycerol and FFAs were similar in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> animals (Table 1). After administration of CL 316,243, the levels of glycerol and FFAs increased in both groups, although to a lesser extent than in the resting state. Again, the concentration of glycerol was significantly lower in the *aP2*<sup>-/-</sup> group ( $0.35 \pm 0.023$  vs.  $0.42 \pm 0.018$  mmol/l;  $P < 0.05$ ). In the postprandial state, a similar trend was also observed for stimulated levels of FFAs, although statistical significance was not reached ( $0.80 \pm 0.074$  vs.  $0.98 \pm 0.093$  mmol/l) (Table 1).

**Lipolysis in isolated adipocytes.** To further confirm that the decreased glycerol levels in *aP2*<sup>-/-</sup> mice were due to diminished release from adipose tissue, we conducted experiments with freshly isolated primary adipocytes. Using different concentrations of dibutyryl cAMP, we observed a significantly reduced release of glycerol and FFAs at submaximal stimulation of lipolysis. At maximal stimulation, release of glycerol and FFAs became equal in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> adipocytes (Fig. 2). A similar right-shift of the dose-response curve in *aP2*<sup>-/-</sup> adipocytes was observed for glycerol and FFAs in experiments using isoproterenol to stimulate lipolysis (data not shown). Finally, we recently established preadipocyte cell lines from *aP2*<sup>+/+</sup> and *aP2*<sup>-/-</sup> mice and, in preliminary experiments, observed significantly reduced lipolytic response in *aP2*<sup>-/-</sup> adipocytes (data not shown). Taken together, these results indicate that the lipolytic response to agents elevating intracellular cAMP is diminished in *aP2*<sup>-/-</sup> versus *aP2*<sup>+/+</sup> adipocytes.

**Gene expression in adipose tissue and isolated adipocytes.** In an attempt to identify mechanisms for the

observed attenuation of lipolysis, we investigated the expression of lipolysis-related genes in white adipose tissue of *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice. Because differences were observed after induction of lipolysis with dibutyryl cAMP, we measured the expression levels of two key molecules of lipolysis, HSL and perilipin, which act at the postreceptor level. The mRNAs for HSL and perilipin were equally expressed in adipose tissue of *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (data not shown). Protein levels of perilipin and HSL were also determined, since perilipin has been reported to be regulated posttranscriptionally (46), and the dosage of HSL can be expected to have a strong impact on lipolysis. Immunoblot analysis demonstrated similar amounts of HSL and perilipin in white adipose tissue of *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (data not shown).

Previously, a strong induction of the *mal1* mRNA has been observed in white adipose tissue of *aP2*<sup>-/-</sup> mice (14). However, the extent of compensation by *mal1* at the protein level has not been determined. To address whether the reduction of lipolysis in *aP2*<sup>-/-</sup> adipocytes is related to loss of a function specific to aP2 or to a general reduction of fatty acid-binding activity, we quantitated the levels of aP2 and *mal1* protein in isolated adipocytes using monospecific antibodies standardized to recombinant murine aP2 and *mal1*. These experiments demonstrated a strong induction of *mal1* protein in *aP2*<sup>-/-</sup> adipocytes, the level approaching that of aP2 in *aP2*<sup>+/+</sup> cells

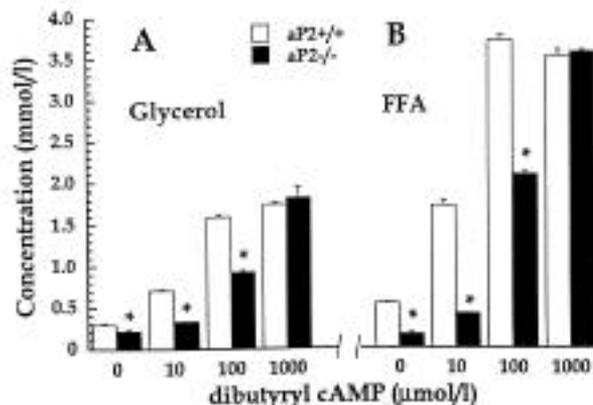


FIG. 2. Lipolysis in adipocytes isolated from *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice. Primary adipocytes were incubated for 1.5 h with the indicated levels of dibutyryl cAMP followed by determination of glycerol (A) and FFAs (B) in the incubation medium. Experiments were performed with cells obtained from animals between 12 and 24 weeks old. A representative experiment (24 weeks) is shown here. \*Significant differences between *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> cells ( $P < 0.05$ ).

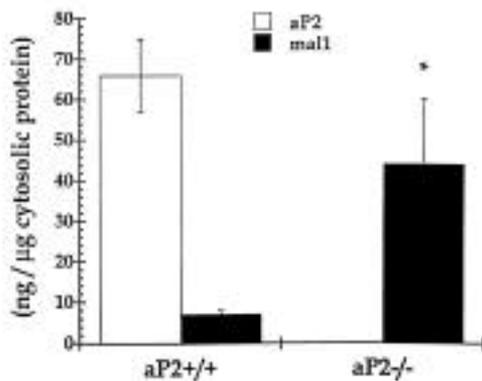


FIG. 3. *aP2* and *mall* protein levels in isolated *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> adipocytes. Protein concentrations were determined by immunoblotting using extracts from primary adipocytes. Samples from four and three independent adipocyte preparations were used for the determination of *aP2* and *mall*, respectively. \*Significant induction of *mall* in *aP2*<sup>-/-</sup> adipocytes ( $P < 0.05$ ).

(Fig. 3). A similar result was obtained when two-dimensional gel electrophoresis was used to determine expression of *aP2* and *mall* in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> adipocytes (data not shown).

**Insulin secretion.**  $\beta$ -Adrenergic stimulation *in vivo* is known to be accompanied by acute secretion of insulin (47,48). The mechanism of  $\beta$ -AR-mediated insulin secretion is not fully understood. Recent studies have shown that a major part of this secretory response is mediated by  $\beta$ -ARs on adipocytes without direct stimulation of pancreatic  $\beta$ -cells (43). Because chronically increased insulin secretion has been proposed to contribute to the development of insulin resistance (23) and *aP2*<sup>-/-</sup> mice are protected from hyperinsulinemia and insulin resistance (14), we wondered whether alterations exist in the insulin secretory response to  $\beta$ -agonists in these animals. Indeed, we observed a profoundly blunted insulin response to CL 316,243 in *aP2*<sup>-/-</sup> mice investigated in the resting state. Whereas the basal insulin levels were similar in both groups, stimulated insulin levels of *aP2*<sup>-/-</sup> mice ( $7.5 \pm 1.05$  ng/ml) reached only 39% of the levels seen in *aP2*<sup>+/+</sup> controls ( $19.4 \pm 1.85$  ng/ml;  $P < 0.0001$ ) (Fig. 4), with similar glucose levels ( $164 \pm 6.3$  vs.  $150 \pm 7.3$  mg/dl, *aP2*<sup>+/+</sup> vs. *aP2*<sup>-/-</sup>;  $n = 13$ ). An even stronger effect was observed after administration of isoproterenol. Insulin levels rose to  $23.3 \pm 2.4$  ng/ml in *aP2*<sup>+/+</sup> mice but only to  $5.7 \pm 0.9$  ng/ml in *aP2*<sup>-/-</sup> animals ( $P < 0.0001$ ) (Fig. 4). This diminished response in the *aP2*<sup>-/-</sup> mice was also reflected in systemic levels of C-peptide, a more accurate indicator of insulin secretion than insulin itself. While basal levels were equal in both genotypes ( $352 \pm 26$  vs.  $370 \pm 18$  pmol/l in *aP2*<sup>-/-</sup> vs. *aP2*<sup>+/+</sup> animals;  $n = 8$ ), C-peptide levels in *aP2*<sup>-/-</sup> animals rose to only ~50% of those measured in *aP2*<sup>+/+</sup> animals ( $1847 \pm 267$  vs.  $3845 \pm 426$  pmol/l;  $P < 0.005$ ;  $n = 7$ ) after administration of CL 316,243.

One potential cause for the observed attenuation of  $\beta$ -AR-mediated insulin secretion in *aP2*<sup>-/-</sup> mice is a general reduction in pancreatic  $\beta$ -cell activity. To test this hypothesis, we performed insulin secretion experiments using alternative insulin secretagogues including arginine (1 g/kg) or a combination of arginine and the sulfonylurea glyburide (0.2 mg/kg). After intraperitoneal administration of arginine, systemic insulin concentrations were similar in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice ( $4.7 \pm 0.41$  vs.  $6.1 \pm 0.94$  ng/ml) (Fig. 4), with similar glucose con-

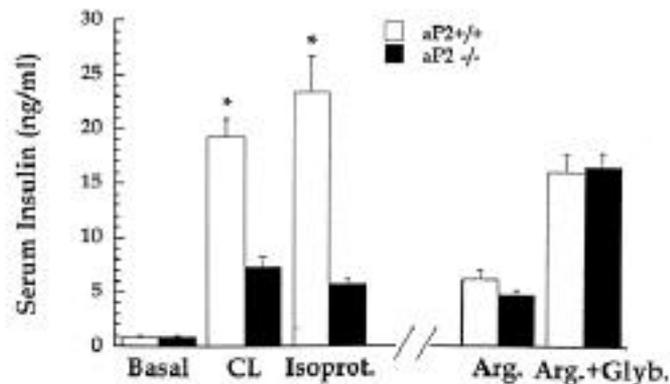


FIG. 4. Insulin secretion in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice. Insulin levels were determined in serum from untreated animals ( $n = 18$ ) and 15 min after intraperitoneal administration of CL 316,243 (CL;  $n = 20$ ), isoproterenol (Isoprot.;  $n = 12$ ), arginine (Arg.;  $n = 7$ ), or a combination of arginine and glyburide (Arg. + Glyb.;  $n = 4$ ). \*Insulin levels were significantly different between *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice after stimulation with CL 316,243 and isoproterenol ( $P < 0.0001$ ).

centrations ( $128 \pm 9.6$  vs.  $124 \pm 5.6$  mg/dl, *aP2*<sup>+/+</sup> vs. *aP2*<sup>-/-</sup>). The combination of arginine and glyburide was more effective, leading to even higher yet similar levels in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice ( $16.6 \pm 1.2$  vs.  $16.1 \pm 1.6$  ng/ml) (Fig. 4).

Because altered insulin secretion might have additional effects on physiologic parameters in the postprandial period compared with the resting period, we also investigated  $\beta$ -AR-mediated insulin secretion in mice under this metabolic condition. As expected, basal insulin levels were elevated in postprandial animals (Table 1). On injection of CL 316,243, blood insulin levels rose in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (Table 1). However, significantly higher insulin concentrations were observed in *aP2*<sup>+/+</sup> compared with *aP2*<sup>-/-</sup> mice ( $8.4 \pm 2.4$  vs.  $3.3 \pm 0.9$  ng/ml;  $P < 0.05$ ).

**Systemic concentrations of insulin secretagogues in response to  $\beta$ 3-adrenergic stimulation.** Experimental elevation of systemic FFAs has been shown to stimulate insulin secretion *in vivo*, making FFAs likely candidates for adipose-derived insulin secretagogues associated with  $\beta$ 3-adrenergic stimulation. Total FFA levels appeared not to be responsible, however, for the difference in insulin secretion between the genotypes in our experiments where, in the resting state, systemic FFA levels were equal or even higher in *aP2*<sup>-/-</sup> vs. *aP2*<sup>+/+</sup> mice (Fig. 1B). Therefore, we tested whether the observed suppression of insulin secretion might be due to altered blood levels in *aP2*<sup>-/-</sup> mice of other known secretagogues. To determine a kinetic profile of insulin and these secretagogues, blood was collected from untreated animals and 5, 10, or 15 min after intraperitoneal administration of CL 316,243. As shown in Fig. 5A, insulin began to rise 5 min after  $\beta$ 3-adrenergic stimulation. The average blood insulin concentration in the *aP2*<sup>-/-</sup> mice was already significantly lower at that point compared with the *aP2*<sup>+/+</sup> controls. Insulin continued to increase up to 15 min after injection, where the difference between the genotypes was most striking (Fig. 5A). Basal blood glucose levels were also lower in *aP2*<sup>-/-</sup> mice compared with *aP2*<sup>+/+</sup> controls ( $142 \pm 8.3$  vs.  $167 \pm 6.4$  mg/dl;  $P < 0.05$ ) (Fig. 5B). After  $\beta$ 3-adrenergic stimulation, no significant difference in blood glucose levels was evident between the two genotypes (Fig. 5B), despite strikingly different insulin concentrations, suggesting a difference in insulin sensitivity.

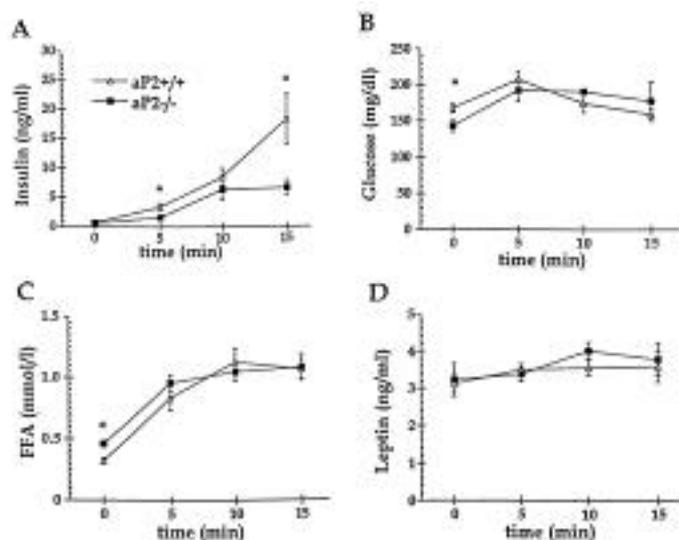


FIG. 5. Blood levels of effectors of insulin secretion. Serum levels of insulin (A), glucose (B), FFAs (C), and leptin (D) untreated or 5, 10, or 15 min after intraperitoneal administration of CL 316,243. Each animal was used for only one time point;  $n = 5-8$  (0, 5, 10 min),  $n = 4-5$  (15 min). \* $P < 0.05$ .

Basal FFA concentrations were moderately but significantly higher in  $aP2^{-/-}$  compared with  $aP2^{+/+}$  mice ( $0.46 \pm 0.03$  vs.  $0.32 \pm 0.02$  mmol/l;  $P = 0.001$ ). FFA levels rose to near-maximal levels 5 min after administration of CL 316,243 and increased only slightly thereafter, with no significant difference between the genotypes (Fig. 5C). Recent studies have shown that leptin can suppress insulin secretion (49–53). To test a potential role of leptin, we also measured serum leptin levels before and after the injection of CL 316,243. Serum leptin levels were similar in both genotypes under basal and stimulated conditions throughout the experiment (Fig. 5D).

Recently published data indicate that the endogenous subtypes of fatty acids differ in their potential to stimulate insulin secretion from pancreatic  $\beta$ -cells (54). Since absence of aP2 might change the composition, and therefore the insulinotropic potency, of FFAs released from adipocytes, we isolated the FFA fraction from serum collected after  $\beta$ 3-adrenergic stimulation and quantified individual fatty acids (Fig. 6). Among the major fatty acid species, a significant difference between  $aP2^{-/-}$  and  $aP2^{+/+}$  mice was observed only for stearic acid (28% decrease in  $aP2^{-/-}$  vs.  $aP2^{+/+}$  mice;  $P < 0.01$ ) and palmitoleic acid (66% increase in  $aP2^{-/-}$ ;  $P < 0.01$ ). Of the less abundant fatty acid species detected in our study, a significant difference was found for *cis*-11-eicosenoic acid (20:1  $\omega$ 9) (30% decrease in  $aP2^{-/-}$ ;  $P < 0.05$ ).

**aP2 expression in pancreatic  $\beta$ -cells.** Because the insulin secretion pattern in  $aP2^{-/-}$  mice is significantly altered, it is critical to determine whether the *aP2* gene is expressed in pancreatic islets. Previous studies have not directly assessed the presence or absence of aP2 in these cells. Therefore, we determined aP2 expression in isolated mouse islets by reverse transcription-PCR. In these experiments, aP2 could be readily detected in white adipose tissue and even liver tissue with low abundance of aP2 (reflecting presence of contaminating adipocytes). However, no expression of aP2 was found in islets (Fig. 7). Integrity of the islet cDNA was confirmed by detection of HNF4 $\alpha$ , a gene known to be expressed in these cells.

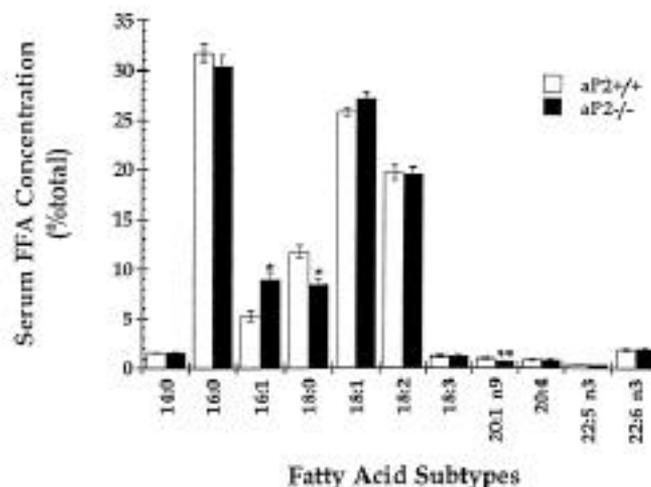


FIG. 6. Serum FFA composition in  $aP2^{-/-}$  and  $aP2^{+/+}$  mice after  $\beta$ 3-adrenergic stimulation. Individual fatty acids were quantitated in the FFA fraction from serum collected 12.5 min after administration of CL 316,243. For palmitoleic acid (16:1), stearic acid (18:0), and *cis*-11-eicosenoic acid (20:1  $\omega$ 9), a statistically significant difference between  $aP2^{-/-}$  and  $aP2^{+/+}$  mice was observed ( $n = 6$ ) (\* $P < 0.01$ ; \*\* $P < 0.05$ ).

## DISCUSSION

In this study, we examined lipolysis and the associated insulin secretion in mice lacking aP2 and their wild-type counterparts. Experiments performed under various physiologic conditions consistently demonstrated an attenuated rise of glycerol in the blood of  $aP2^{-/-}$  animals in response to  $\beta$ -adrenergic stimulation (Fig. 1; Table 1). Furthermore, a right-shift in the dose response of glycerol and FFA release to stimulation by dibutyl cAMP was observed in primary adipocytes isolated from  $aP2^{-/-}$  mice (Fig. 2). While these experiments did not specifically address the extent that FFA flux from adipose tissue to other major metabolic organs is altered in  $aP2^{-/-}$  mice, the results clearly indicate a reduction of the lipolytic response in  $aP2^{-/-}$  adipocytes, demonstrating a critical function of aP2 in the regulation of fatty acid metabolism.

Another critical question is whether the observed lipolysis phenotype might be due to general loss of cytosolic fatty acid-binding activity, a condition that might impair efflux of

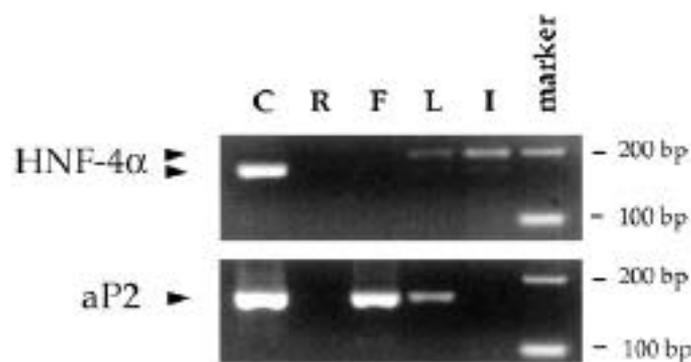


FIG. 7. Detection of aP2 mRNA in islets and other tissues. Mouse islet cDNA and cDNA from control tissues was investigated by reverse transcription-PCR using primer pairs specific for aP2 and HNF4 $\alpha$  (control). The data shown are representative for three independent experiments. The HNF4 $\alpha$  PCR detects two splice isoforms. C, cDNA clone; R, reagent control; F, white adipose tissue; L, liver; I, islet.

FFAs. To address this issue, we determined the compensatory increase in mall protein in *aP2*<sup>-/-</sup> adipocytes and found that it approaches levels similar to those of *aP2*<sup>+/+</sup> adipocytes (Fig. 3). This result does not support reduced abundance of general fatty acid-binding activity as a cause of the observed phenotype. Rather, it suggests that *aP2* exhibits specific functional and qualitative differences from mall in its efficiency as a cofactor of lipolysis. Such a specific difference is not likely to be related to fatty acid-binding affinities, which are similar between these two members of the FABP family (55,56). Since both proteins are quite different in their surface charge pattern (57), one might speculate that they interact differently with intracellular targets such as phospholipid membranes or proteins.

The mRNA and protein levels of both HSL and perilipin in white adipose tissue of *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> animals were similar, arguing against a quantitative defect in the key components of the lipolytic machinery. It is possible, however, that the reduction in lipolysis might be caused by posttranslational events. For example, earlier reports have suggested that HSL activity might be directly or indirectly regulated at the posttranslational level by intracellular FFAs (58–61). Further experiments with a suitable in vitro model will determine the mechanisms responsible for the observed attenuation of lipolysis in *aP2*<sup>-/-</sup> adipocytes.

Stimulation of lipolysis with  $\beta$ -AR-specific agonists such as CL 316,243 and isoproterenol is accompanied by an acute increase in blood insulin (47,48,62). Surprisingly, this insulin response was profoundly reduced in *aP2*<sup>-/-</sup> mice (Fig. 4; Table 1). A similar reduction of C-peptide levels in *aP2*<sup>-/-</sup> mice after  $\beta$ -adrenergic stimulation indicates that the defect is, at least partially, at the level of insulin secretion. This decreased responsiveness is unlikely to be through an indirect mechanism such as generally diminished activity of pancreatic  $\beta$ -cells (63–65), since arginine or a combination of arginine and glyburide stimulated insulin secretion to a similar extent in *aP2*<sup>-/-</sup> and *aP2*<sup>+/+</sup> mice (Fig. 4). Also, the reduced response is probably not related to glucose, which is a weak insulin secretagogue in the mouse strain used (C57Bl/6) (66). We also tested whether a previously unrecognized expression of *aP2* in pancreatic islets might be a factor in this reduced response. As shown in Fig. 7, there is no detectable *aP2* mRNA present in islets, excluding the possibility of a direct impact of *aP2* deficiency at that site.

Previous studies have suggested that the signal for  $\beta$ -AR stimulation of insulin secretion originates from adipocytes (43) and that it involves fatty acids (25,54,67,68). However, the strikingly different insulin response seen in the *aP2*<sup>-/-</sup> mice in our study occurred under experimental conditions in which significant differences in blood concentrations of total FFAs compared with wild-type controls were not evident (Fig. 5). This prompted us to examine specific fatty acids, since recently published experiments using perfused rat pancreas showed that the insulin secretory potential of individual fatty acid species is quite different, increasing with length and saturation of the hydrocarbon chain (54). In our experiments, we did not observe a significant quantitative difference between the genotypes for most of the circulating fatty acid species investigated after  $\beta$ -adrenergic stimulation. Interestingly, in *aP2*<sup>-/-</sup> mice a significant decrease was observed of stearic acid, which had the strongest secretory activity in the perfused pancreas experiments mentioned above. *cis*-11-Eicosenoic

acid, a minor fatty acid species, was also decreased (Fig. 6). Reciprocally, an increase in palmitoleic acid, which had little secretory potential in the perfused pancreas experiment, was evident in the *aP2*<sup>-/-</sup> mice. It is worth noting that the change in stearic acid constituted only a small fraction (<4%) of total FFA amount, and the insulinotropic potency of *cis*-11-eicosenoic acid has not been determined. Therefore, it is not clear whether these alterations are physiologically relevant and might account for the profoundly reduced insulin response in *aP2*<sup>-/-</sup> mice.

In this study, we observed decreased release of both glycerol and FFAs in response to dibutyl cAMP stimulation in *aP2*<sup>-/-</sup> adipocytes and decreased plasma glycerol but not FFA levels in response to  $\beta$ -adrenergic stimulation in whole animals. This difference is most likely due to the more ubiquitous and complex nature of fatty acid versus glycerol metabolism in vivo, where plasma FFA levels reflect the net outcome of adipocyte lipolysis and lipoprotein lipase action as well as uptake and re-esterification in many tissues, including adipose. It is possible that other experimental conditions such as prolonged fasting or use of alternative signals to stimulate lipolysis at submaximal doses might allow detection of decreased plasma FFA levels in *aP2*<sup>-/-</sup> mice. Regardless, it is clear from the data shown here that under conditions where systemic FFA levels are similar, a dramatic difference is still detectable in insulin secretion in response to lipolytic stimuli in *aP2*-deficient animals. While it is possible to argue that the net local FFA input to the pancreatic islets might have been altered despite the apparent similarity in serum FFA concentrations, the possibility of additional insulinotropic molecules that are released from adipose tissue in response to  $\beta$ -adrenergic stimulation should be recognized. The secretion of such factors would correlate with lipolysis, and therefore with the presence of *aP2*, but not necessarily with fatty acid efflux from adipose tissue. Future experiments are needed to confirm the existence and identity of these signals. In any case, the observed endocrine alteration qualifies the *aP2*<sup>-/-</sup> mice as an excellent tool for understanding the relationship between adipose tissue and the endocrine pancreas.

Interesting possibilities emerge in view of the observed impact of *aP2* deficiency on insulin secretion. First, these results provide strong support for the notion of an endocrine axis between adipose tissue and the pancreas, demonstrating that a specific and isolated defect of adipose tissue can have a profound effect on the latter organ. Second, since hyperinsulinemia can lead to peripheral insulin resistance, *aP2*<sup>-/-</sup> animals might be protected from the development of obesity-induced diabetes, at least in part, through a reduction in insulin secretion triggered by adipocyte products. Currently, the mechanisms underlying increased peripheral insulin sensitivity in the absence of *aP2* are not fully understood. Further characterization of these pathways might therefore provide important insights into the pathophysiology of type 2 diabetes and facilitate the development of novel therapeutic targets.

**Note added in proof.** During the review of this manuscript, Coe et al. (*J Lipid Res* 40:967–972, 1999) also reported defective lipolysis in *aP2*<sup>-/-</sup> mice. In a separate study, Shen et al. (*Proc Natl Acad Sci U S A* 96:5528–5532, 1999) reported a physical interaction between *aP2* and HSL, suggesting a potential mechanism for the involvement of *aP2* in lipolysis.

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