Expression of the adipocyte fatty acid-binding protein in streptozotocin-diabetes: effects of insulin deficiency and supplementation

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Abstract The adipocyte fatty acid-binding protein, aP2 or ALBP, is an abundant cytosolic protein postulated to function in binding and intracellular transport of long-chain fatty acids. In this report, we investigated levels of aP2 mRNA and protein and transcriptional activity of the aP2 gene in tissues from streptozotocin-diabetic rats at different time periods following the induction of diabetes. An average 75% decrease in mRNA for aP2 (relative to mRNA for β-actin) was observed in all diabetic rats at 7 days post-STZ injection. Insulin supplementation rapidly (2 h) restored aP2 mRNA and the insulin effect was cycloheximide-sensitive. Nuclear transcription assays measured a 60% decrease in transcription of the aP2 gene in diabetic rats that was reversed by insulin administration. Levels of aP2 protein were still high, in some cases, 1 day after the decrease in mRNA levels consistent with a long half-life of the protein. Decreases in aP2 protein were rapidly reversed by insulin administration. There were no changes in aP2 protein in the absence of changes in aP2 mRNA supporting a pretranslational mechanism of regulation. The decrease in aP2 mRNA was delayed in onset when compared with the rapid decline (at day 2 of diabetes) of mRNA for the lipogenic enzyme, fatty acid synthase, and with the accelerated depletion of adipose tissue lipid. Adipose tissue weight and lipid content had decreased by more than 80% 3 days before any significant changes in aP2 expression were observed. Changes in aP2 could not be related to changes in the levels of circulating fatty acids that regulate aP2 expression in vitro. The study indicated 1) that insulin deficiency and supplementation can regulate expression of aP2 in vivo and 2) that changes in aP2 levels are unlikely to contribute to the abnormalities of fatty acid metabolism in adipose tissue from diabetic rats.—Melki, S. A., and N. A. Abumrad.


Supplementary key words adipose tissue • fatty acid-binding protein • insulin • streptozotocin diabetes

The adipose lipid-binding protein (ALBP or aP2) belongs to a family of related cytosolic proteins thought to bind (1) long chain fatty acids (FA) and possibly to shuttle them to sites of FA metabolism (2-4). Consistent with this, a large induction of aP2 is observed upon differentiation of preadipocytes in culture coinciding with the increase in FA esterification rate (5-7). This induction is dependent on the presence of adipogenesis-promoting hormones, insulin and insulin growth-factor I, IGF-1 (8, 9). In addition to its postulated roles in intracellular binding and shuttling of FA, there is evidence that aP2 is phosphorylated by the insulin receptor tyrosine kinase leading to the suggestion that it might be involved in the insulin-signaling pathway in adipocytes (10, 11).

There is little information concerning regulation of aP2 expression in vivo. Such information is important for evaluating the physiological role of aP2. In particular, the effect of diabetes on aP2 expression would be of interest as this condition is associated with marked disorders of FA metabolism. Diabetes is characterized by an increase in FA mobilization and by a progressive and marked wasting of triglyceride stores in adipose tissue. We examined aP2 expression in streptozotocin (STZ) diabetic rats at different times after induction of diabetes and after insulin supplementation and withdrawal. Metabolic measurements were also carried out under the same conditions. Our data indicated that levels of aP2 mRNA and protein are markedly decreased by diabetes and this decrease is rapidly reversed by insulin supplementation. However, the decrease in aP2 gene expression was delayed when compared with diabetes-induced alterations in adipose tissue.

Abbreviations: FA, fatty acid; STZ, streptozotocin; i.p., intraperitoneal; aP2, adipose fatty acid-binding protein; EF1, enhancing factor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FAS, fatty acid synthase; GTC, guanidinium isothiocyanate; MOPS, 3-(N'-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate.

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RESEARCH DESIGN AND METHODS

Materials

Streptozotocin (STZ) was obtained from Calbiochem (La Jolla, CA). The insulin used was either regular insulin or NPH purified pork isophane insulin suspension, both from Squibb Novo (Princeton, NJ). Guanidinium isothiocyanate and film (X-OMAT, AR) were from Kodak (Rochester, NY). Cesium chloride was from Fisher Scientific (Fair Lawn, NJ). The supported nitrocellulose filters were from Schleicher and Schuell (Keene, NH). The random-primed labeling kit and 32P-dCTP were obtained from Amersham (Arlington Heights, IL). All other reagents, unless noted, were obtained from Sigma (St. Louis, MO). The respective cDNAs for aP2, fatty acid synthase (FAS), β-actin, and enhancing factor 1 (EF1, ref. 12) were kindly provided by Drs. Howard Green, Marc Magnusson, Paul Grimaldi, and Linda Sealy, respectively. Antibody against aP2 (13) was a generous gift from Dr. David Bernlohr.

Diabetes induction and insulin treatment

Sprague-Dawley male rats (150-200 g) were obtained from Sasco (Omaha, NE). They were fasted for 12-14 h before receiving an intraperitoneal (ip) injection of streptozotocin (STZ, 85 mg/kg body weight) in 0.2 ml of 100 mM Na citrate buffer, pH 4.5. Control rats followed the same treatment except that STZ was omitted from the citrate buffer.

To study the effect of insulin treatment on aP2 expression, diabetic rats were given i.p. injections that consisted of 2 U of regular insulin with 2 U of NPH insulin and were killed at the times indicated. To study the effects of insulin withdrawal, regular insulin only was administered in two injections i.p. (2 U followed by 1 U 2 h later). The rats were killed by guillotine at the times indicated after the last insulin injection. All protocols were approved by the animal care committee at Vanderbilt University.

Measurement of plasma glucose, fatty acid, and insulin

Blood samples were obtained at the time of killing. Plasma was recovered by spinning the heparinized samples at 2000 g. Aliquots were then used for measurements of insulin, by radioimmunoassay using a kit from Diagnostic Products Corporation (Los Angeles, CA), glucose (Beckman glucose analyzer), and fatty acid by the 63 nickel assay of Ho and Meng (14).

RNA isolation and analysis

The epididymal fat pad was rapidly dissected out and homogenized in guanidinium isothiocyanate (GTC). Isolation of total RNA was done using the GTC-cesium chloride method (15). For Northern analysis, 25 µg total RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel. Ethidium bromide was added to each sample (0.01 µg/ml) to monitor RNA integrity, the uniformity of sample loading, and efficiency of transfer onto a nylon-supported nitrocellulose membrane. Prehybridization and hybridization with the various cDNA probes were done at 43°C under the same conditions: 5 × saturated sodium citrate (SSC, 3 M NaCl, 0.3 M Na citrate), 50 mM Na phosphate, 1 mM Na pyrophosphate, 25% formamide, 10% dextran sulfate, 5 × Denhart (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% Pentax fraction IV BSA), 0.1% sodium dodecylsulfate (SDS) 0.05% Nonidet NP40, 0.1 mg/ml Torula RNA, and 0.1 mg/ml salmon sperm DNA. The various cDNA probes were labeled using random-prime labeling and [32P]dCTP. Nonspecific binding was removed by washing the membrane at 43°C twice with 3 × SSC, 0.1% SDS buffer and then once or twice with 0.1 × SSC, 0.1% SDS. Signal for aP2 mRNA was quantitated by scanning films from multiple exposures (LKB Ultrascan XL). It was normalized to mRNA for β-actin, EF1, or GAPDH and related to mRNA from control tissue, with all mRNA probed on the same gel.

Tissue extraction and immunoblot analysis

Fat pads were washed with ice-cold phosphate-buffered saline (137 mM NaCl, 1.5 mM KH2PO4, 7 mM Na2HPO4, and 2.7 mM KCl) and homogenized in a tissue grinder (clearance of 0.13-0.18 mm, Thomas Scientific, Swedesboro, NJ). The homogenization buffer (pH 7.5) contained Tris-HCl (20 mM), EDTA (1 mM), and phenylmethane sulfonylfluoride (0.1 mM). The extract was centrifuged at 8000 g for 5 min at 5°C and then at 100000 g for 1 h at 5°C. The infranatant containing soluble proteins was stored at -70°C until use. Protein concentration in the extract was measured using the Bio-Rad protein assay with bovine serum albumin as a standard. Immunodetection of aP2 was carried out essentially as described by Bernlohr et al. (13). One hundred µg of extracted protein was subjected to SDS electrophoresis on a linear 5-15% polyacrylamide gel and then to transfer (12-15 h at 4°C) onto a supported nitrocellulose membrane. Nonspecific binding was blocked by keeping the membrane for 3 h at 23°C in buffer (TNB) consisting of 0.1% Brij-58, 10 mM Tris-HCl, pH 7.4, and 0.5 M NaCl. Antiserum to aP2 was added (23°C, 14-18 h) at a 1:500 dilution in TNB, then the membrane was washed 3 times with TNB and then 3 times in Tris-buffered saline (100 mM Tris, 0.9% sodium chloride, pH 7.5) containing 0.1% (v/v) Tween 20. Incubations with biotinylated antibody and horseradish peroxidase substrate were done according to the Vectastain procedure (Vector Lab., Burlingame, CA).

Nuclear transcription assay

For these experiments, 10-day diabetic rats were given an injection of regular insulin (2 U/rat) or of saline and...
were killed 2 h later. Fat pads were excised, dissected from blood vessels, and incubated for 5 min at 37°C with collagenase (1 mg/ml). Tissue pieces were then recovered in 10 ml of buffer containing 10 mM Tris (pH 7.4), 3 mM MgCl₂, 10 mM NaCl and were homogenized manually using a Dounce homogenizer (clearance B). The homogenate was kept on ice for 10 min. The fat cake was then removed and NP40 was added to a final concentration of 0.5%. Nuclei were then pelleted at 800 x g for 5 min, washed with cold buffer, with and then without NP-40. The pellet was resuspended in 100 µl of buffer containing 50 mM Tris (pH 8), 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA. Pellets were frozen at −70°C until use. Transcriptional assays followed the methodology of Doglio et al. (16). Briefly, nuclei (10⁷) were incubated with 200 µCi of [α-³²P]UTP. The ³²P reaction products (10⁷ cpm) were hybridized for 48 h at 65°C to Hybond-C membranes (Amersham) with 10 pg of the denatured cDNA probes.

Calculations

Data from metabolic measurements were compared using analysis of variance with differences between groups identified by the Newman-Keuls test. The analysis of Northern blots was performed by an unpaired t test as one experimental group was directly compared to a control group. Differences were considered significant at a P < 0.05 level.

RESULTS

Metabolic measurements at different days after induction of diabetes

Table 1 shows that rats injected with STZ and maintained without insulin did not gain weight over the 10-day period of experimental diabetes. The rats weighed an average of 172 ± 6 g before STZ injection and 170 ± 11 g at day 10 after the injection. Body weight of diabetic rats at the time of killing was about 34% less than that of control rats, 260 ± 5 g. These changes are similar to those previously described by others for STZ-diabetic rats (17, 18).

Streptozotocin-treated rats had a blood glucose higher than 400 mg/dl. Plasma insulin fell from 50 µU/ml in control rats to 4 µU/ml at day 2 and remained at this low level for the remainder of the study period. Blood fatty acids were markedly increased in diabetic rats (1.5 mM versus 0.45 mM in control rats) and levels were similar at 2, 4, 7, and 10 days after induction of diabetes. Fat pad weight and lipid content decreased rapidly after STZ injection so that by day 4 both amounted to less than 20% of that from control rats and they remained at about this value for the remainder of the study period. Treatment with a combination of 2 U each of regular and NPH insulin raised plasma insulin to 110 µU/ml at 6 h after the injection. Blood glucose and fatty acids (measured at 6 h) were lowered but remained significantly higher than those of control rats (Table 1). Administration of short-acting regular insulin without NPH insulin (2 U followed by 1 U, 2 h later) increased plasma insulin to about 100 µU/ml at 2 h after the last injection, but levels were back down to less than 3 µU/ml at 6 h (data not shown). Diabetes decreased the total RNA recovered per fat pad from about 110 µg per pad to 60 and 65 µg per pad from diabetic rats without or with insulin treatment (6 h), respectively.

aP2 mRNA at various times after induction of diabetes

Fig. 1 illustrates the changes in aP2 mRNA in diabetic rats kept without insulin. No significant change was observed at day 2 of diabetes, however a decrease was apparent in 30% of the rats tested at day 4 and in 100% of the

| Table 1. Metabolic parameters in streptozotocin-diabetic rats |
|------------------|------------------|
| Rat Weight*      | Pad Weight       |
| % of control     | Glucose (mg/dl)  | Insulin (µU/ml) | FFA (mM) |
| Control          | 100              | 100             | 137 ± 7  | 50 ± 6  | 0.5 ± 0.06 |
| Diabetic Day 2    | 89 ± 2           | 67 ± 3          | 410 ± 20 | 4 ± 1   | 1.5 ± 0.36 |
| Diabetic Day 4    | 78 ± 4           | 30 ± 3          | 425 ± 18 | 3.9 ± 1 | 1.3 ± 0.5  |
| Diabetic Day 7    | 66 ± 4           | 18 ± 2          | 510 ± 22 | 3.4 ± 1 | 1.4 ± 0.4  |
| Diabetic Day 10   | 64 ± 2.5         | 7 ± 1           | 523 ± 40 | 2.5 ± 0.5 | 1.5 ± 0.2 |
| Diabetic Day 10 + insulin* | 65 ± 5         | 9 ± 2           | 404 ± 70 | 110 ± 25 | 1.0 ± 0.30 |

Measurements of plasma glucose, insulin, and nonesterified fatty acid are described in detail under Experimental Procedures. Data are means from at least three experiments (30 rats per experiment) and are shown ± their standard errors (SE). Differences between control and diabetic rats were statistically significant for all parameters shown (P < 0.05 < 0.001).

*Male Sprague-Dawley rats (150–200 g) were made diabetic (day 0) by one injection of streptozotocin i.p. (85 mg/kg BW). At time of killing, control rats weighed 260 ± 5 g. Fat pad weight was 1.9 ± 0.5 g with lipid constituting 85% of the weight.

*Insulin was administered i.p with saline (2 units of regular insulin and 2 units of NPH intermediate acting insulin). Rats were killed 6 h later. Levels of aP2 mRNA in 10-day diabetic rats, before and after insulin, are shown in Fig. 1.
rats tested at day 7 (Fig. 1, A). In contrast to aP2, mRNA levels for fatty acid synthase, FAS, included for comparison, had markedly declined by day 2 of diabetes. The changes in aP2 mRNA were not associated with changes in levels of blood glucose, FA, or insulin which were similar at days 2, 4, 7, and 10 after STZ injection (Table 1). The changes in aP2 mRNA also did not coincide with the decline in wet weight or lipid content of the fat pad which achieved nearly maximal decreases by day 4 after STZ induction, 3 days before any significant change in aP2 mRNA was observed. The decrease in aP2 mRNA was not due to a generalized nonspecific decrease of cellular mRNA as all levels reported for aP2 mRNA were normalized for those of β-actin and/or EF1. When aP2 mRNA level was expressed per fat pad as a way of relating changes to adipocyte number, which is not altered by diabetes, a pad from a diabetic rat as compared with a pad from a control rat had 0.2 (± 0.03) of the aP2 mRNA signal as contrasted with 0.94 (± 0.13) and 0.82 (± 0.15) of the β-actin and EF1 mRNA signals, respectively.

Effect of insulin supplementation on aP2 mRNA

The time course of insulin’s effect on aP2 expression in 10-day diabetic rats is shown in Fig. 2. Levels of aP2 mRNA increased to about 75% of those observed in tissues from control rats as early as 2 h after an i.p. injection of insulin. No further increases in aP2 mRNA were observed at 6 and 24 h after insulin administration. Fig. 2B shows the changes in aP2 mRNA normalized for those in EF1 mRNA and expressed as percent of control values.

Effect of cycloheximide on insulin induction of aP2 mRNA

Cycloheximide, a potent protein synthesis inhibitor, was administered i.p. (1 mg/100 g body weight) prior to insulin treatment to investigate whether restoration of aP2 mRNA by insulin was dependent on ongoing protein synthesis. As seen in Fig. 3, cycloheximide treatment abolished insulin’s ability to increase aP2 mRNA. Blood insulin levels in cycloheximide-treated rats were high and similar to those in rats that did not receive cycloheximide. In addition, no effect of cycloheximide on mRNA for GAPDH was observed in the absence or presence of insulin.

Decay of aP2 mRNA after its induction by insulin

Although aP2 mRNA in tissues from diabetic rats increased rapidly with insulin supplementation, it responded slowly to insulin withdrawal after a short treatment. As shown in Fig. 3, aP2 mRNA remained high 48 h after the last of two injections of regular insulin. In contrast, mRNA for FA synthase was undetectable 24 h after the last injection (data not shown). Under these conditions, insulin had disappeared from the blood and measured less than 3 μU/ml by 6 h after the second insulin administration. Therefore, induction of aP2 by insulin treatment was independent of the sustained presence of high circulating levels of insulin. This was consistent with the delayed decrease of aP2 mRNA following diabetes induction.
**aP2 protein levels in untreated and insulin-supplemented diabetic rats**

Levels of aP2 protein remained unaltered in 40% of the tissues where mRNA for aP2 had decreased by more than 70%. However, decreases in aP2 protein levels were reversed 6 h after an ip insulin injection (Fig. 4). There were no changes in aP2 protein and no effects of insulin on protein levels in the absence of effects on aP2 mRNA that supported a pretranslational mechanism.

**Effect of diabetes on transcription of the aP2 gene**

The rate of aP2 gene transcription was measured in tissues from diabetic rats at day 10 of diabetes before and 2 h after one injection of regular insulin (2 U). Transcription of the aP2 gene (Fig. 5) normalized to β-actin, was 60% lower in nuclei isolated from diabetic rats as compared to that in tissues from healthy littermates. This decrease was similar in magnitude to that described for aP2 mRNA (75%). The decrease in transcription of the
Fig. 4. aP2 protein levels in tissues from streptozotocin-diabetic rats without and with insulin supplementation. Adipose tissue extracts were prepared from control, 2-, and 10-day diabetic rats and from 10-day diabetic rats treated with insulin for 6 h as in the legend to Fig. 2. One hundred µg of soluble protein was subjected to SDS electrophoresis on a linear 5-15% polyacrylamide gel and blotted onto a nitrocellulose filter in buffer with 20 mM Tris base, 150 mM glycine, 20% methanol, and 0.1% SDS. Immunodetection of aP2 was done with anti-aP2 antibody as detailed in the text. Data shown are representative of four more experiments.

aP2 gene did not reflect a general decrease in transcription rate, as it largely exceeded the small decline in transcription (about 5%) for β-actin. Insulin supplementation (Fig. 5) to diabetic rats increased the rate of aP2 gene transcription about 4-fold at 2 h after the injection.

**DISCUSSION**

The present work examined expression of the adipose fatty acid binding protein (aP2) in tissues from streptozotocin-diabetic rats. Our results indicated that diabetes produced large decreases in aP2 gene transcription and in levels of aP2 mRNA and protein. A previous study by Flier et al. (19) reported smaller decreases in aP2 mRNA (25%) in adipose tissue from diabetic rats. However, the rats were given a low dose of streptozotocin (40 mg STZ/kg BW) and may have had significant residual insulin.

A delay of 7 days was necessary for the decrease in aP2 mRNA to occur. This was not related to the metabolic status of the animals as 2-, 4-, 7-, and 10-day diabetic rats presented the same metabolic profile, yet aP2 mRNA was markedly different. The need for a long period of insulin deficiency in order to observe a decrease in aP2 mRNA was consistent with its slow decay in tissues from diabetic rats after a short treatment with regular insulin. aP2 mRNA from tissues of 10-day diabetic rats supplemented with insulin increased approximately 3-fold within 2 h. However, the effect of the insulin injection took several days to decay.

The slow decline in aP2 mRNA contrasted with the rapid disappearance of mRNA for lipogenic enzymes (FAS, Fig. 1 and acetyl CoA carboxylase, data not shown) and with the accelerated wasting of adipose tissue. The findings suggest that changes in aP2 levels did not contribute to the abnormalities of fatty acid metabolism in adipose tissue from diabetic animals. However, alterations in aP2 function could have played a role. For example, decreased aP2 phosphorylation, consequent to insulin absence, could reduce capacity of aP2 for binding fatty acids (10, 11). In turn, this could promote fatty acid release from adipocytes and result in a decrease in fatty acid esterification. Finally, persistence of aP2 until most of adipose lipid is mobilized might emphasize its role as an acceptor of FA in the cytoplasm, acting to protect cellular proteins under conditions of high free fatty acids.

Insulin's effect to restore levels of aP2 mRNA was inhibited by cycloheximide. This drug potentially can have multiple nonspecific toxic effects. However, the lack of cycloheximide effect on GAPDH mRNA under the same conditions, would argue against such effects and would suggest that insulin action on aP2 mRNA was dependent on protein synthesis. As effects of insulin on aP2 mRNA probably reflected effects on gene transcription, the data would suggest that insulin induces the synthesis of protein(s) that regulate transcription of the aP2 gene. These regulatory proteins would have a long half-life since aP2 mRNA persisted, after insulin decay, for periods of time that largely exceeded its turnover time (half-time of 12-14 h, ref. 20).

Our data do not differentiate between direct and indirect effects of insulin on aP2. It is possible that the effects of insulin deficiency and supplementation were mediated by other factors. Long-chain FA (7, 21) in the unprocessed form (22) have been shown to regulate aP2 mRNA in vitro. However, fatty acids induce aP2 gene expression in vitro, and are significantly increased by STZ diabetes (Table 1) so they would be expected to increase rather than decrease aP2 mRNA in diabetic rats. It is possible that the high circulating levels of fatty acids and/or glucose might have contributed to the delay in decay of aP2 mRNA with diabetes. However, changes in

![Graph showing transcription rate of aP2 gene](image)
levels of these substrates do not explain the decrease in aP2 mRNA or its increase after insulin administration. A recent report (23) described a fall in IGF-1 levels in STZ diabetes that was restored by insulin treatment. Since IGF-1 has been shown to be more potent than insulin in inducing aP2 gene expression in vitro (8, 9), it is possible that changes in IGF-1 levels mediated the in vivo effects of insulin deficiency and supplementation on aP2. It is worth noting that regulation of aP2 gene expression in cultured adipocytes by insulin and IGF-1 is observed at the preadipocyte stage when cells are still lacking lipid and lipid-synthesizing enzymes (8, 9). These same factors do not regulate aP2 in mature adipocytes. One hypothesis that can be presented in order to explain our data and to reconcile our findings with those described earlier in vitro is that prolonged insulin deficiency in vivo and the associated depletion of adipocyte lipid generate a cellular state similar to that of the preadipocyte. It is likely that the changes we observed in aP2 mRNA reflect loss of differentiation-linked gene expression. Berger et al. (24) and Kahn et al. (18) reported a selective decrease in the levels of the glucose transporter, Glut 4, in adipocytes from STZ-diabetic rats at day 7 of diabetes and this decrease was reversed by insulin administration. In contrast to the decrease in Glut 4, a transporter that is specific to adipose and muscle tissues, the authors reported no alteration in the levels of the ubiquitous glucose transporter, Glut 1 (24). Consistent with this we found that, at day 7 after STZ injection, adipose tissue homogenates from diabetic rats exhibited a selective decrease in the activity of the palmitoyl-CoA synthase while that of arachidonoyl-CoA synthase was unaltered (N. A. Abumrad, unpublished observations). Palmitoyl-CoA synthase is differentiation-dependent in cultured adipocytes while arachidonoyl-CoA synthase is not (22). All of the above suggests that the sustained presence of insulin is necessary to maintain not only the activities of lipogenic enzymes and lipid storage but also the differentiated state of the adipocyte.

In summary, our studies showed clear effects of insulin deficiency and supplementation on aP2 gene expression in vivo. The metabolic implications of the regulatory effects are uncertain as they were not exerted within the same time frame as the major metabolic changes of adipose tissue. The effects of insulin on aP2 gene expression in vivo support observations made in vitro suggesting an important role of insulin or IGF-1 in promoting and maintaining differentiation of adipocytes (8, 9). The data would also be in accord with a function of FA-binding proteins in cell growth and differentiation (25, 26).

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