THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 43, Issue of October 22, pp. 44320–44326, 2004
Printed in U.S.A.
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gAd-globular Head Domain of Adiponectin Increases Fatty Acid Oxidation in Newborn Rabbit Hearts*

Received for publication, January 13, 2004, and in revised form, June 29, 2004
Published, JBC Papers in Press, July 21, 2004, DOI 10.1074/jbc.M400347200

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Adiponectin is an adipocyte-derived hormone that has a number of metabolic effects in the body, including the control of both glucose and fatty acid metabolism. The globular head domain of adiponectin, gAd, has also been shown to increase fatty acid oxidation in skeletal muscle. Within days after birth, a rapid increase in fatty acid oxidation occurs in the heart. We examined whether adiponectin or gAd plays a role in this maturation of cardiac fatty acid oxidation. Plasma adiponectin increased in newborn rabbits following birth: 1.2 ± 0.3 μg/ml in 1-day-old, 6.8 ± 1.8 μg/ml in 7-day-old, and 45 ± 5 μg/ml in 6-week-old rabbits. Because plasma insulin levels decrease and remain low throughout the suckling period, and because this decrease may contribute to the maturation of fatty acid oxidation, we examined the effects of adiponectin and gAd on fatty acid oxidation in isolated perfused 1-day-old rabbit hearts in the presence or absence of 100 microunits/ml insulin. Adiponectin (10 μg/ml) did not alter fatty acid oxidation in the presence of insulin. In the absence of insulin, the addition of recombinant gAd (1.5 μg/ml) increased fatty acid oxidation compared with control (129 ± 18 *versus* 66 ± 11 nmol·g dry weight $^{-1}$·min^{-1}, respectively ($p < 0.05$). In 7-day-old hearts, where fatty acid oxidation rates were 5-fold higher than 1-day-old hearts, gAd did not alter fatty acid oxidation rates. The increase in fatty acid oxidation in 1-day-old hearts occurred independently of changes in 5′-AMP-activated protein kinase, acetyl-CoA carboxylase, or malonyl-CoA. The effect of gAd on fatty acid oxidation was reversed in the presence of 100 microunits/ml insulin. These results suggest that a decrease in plasma insulin and increase in gAd are involved in the increase of cardiac fatty acid oxidation in the immediate newborn period.

Adiponectin is an adipocyte-derived polypeptide hormone of ~30-kDa (1). It has a signal sequence at the N terminus, a small nonhelical region, a stretch of repeated collagens, and a C-terminal globular head domain (gAd) that makes up for the majority of the protein (2). Recently, gAd has been shown to increase fatty acid oxidation in muscle and cause weight loss in mice (3). Yamauchi *et al.* (4) demonstrated that gAd and full-length adiponectin stimulate 5′-AMP-activated protein kinase (AMPK)[1] in skeletal muscle. Activation of AMPK increases the phosphorylation of acetyl-CoA carboxylase (ACC), fatty acid oxidation, glucose uptake, and lactate production in C2C12 myocytes. When administered *in vivo*, both full-length adiponectin and gAd stimulated AMPK and increased the phosphorylation of ACC in the mouse soleus muscle (4). Moreover, stimulation of AMPK with full-length adiponectin also reduces gluconeogenesis in the liver and plasma glucose (4). In agreement with these findings, Tomas *et al.* (5) reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC. Recently, Yamauchi *et al.* (6) cloned complementary DNAs encoding two adiponectin receptors, AdipoR1 and AdipoR2. The authors showed that AdipoR1 is abundantly expressed in skeletal muscle and heart, whereas AdipoR2 is the predominant form in the liver. The expression of AdipoR1 in C2C12 myocytes increased fatty acid oxidation and glucose uptake on stimulation with gAd, and these effects were partially inhibited by dominant negative AMPK. This suggests that AMPK is involved in the signaling through the adiponectin receptor.

Cardiac energy in the form of ATP is primarily supplied from the oxidation of fatty acids and glucose in the adult heart. In contrast, in the fetal heart, lactate oxidation and glycolysis are the preferred sources for ATP production (reviewed in Refs. 7–9). As the newborn heart matures, fatty acid oxidation increases and becomes the dominant oxidative substrate for the heart. The mechanism for this switch in energy substrate preference is because of a combination of changes of nutrition and environment, as well as direct subcellular changes within the myocardium.

Plasma insulin decreases and glucagon increases in the immediate postnatal period in most species including humans, rats, rabbits, sheep, and pigs (10, 11). Perinatal decreases in insulin and increases in glucagon have been suggested to be the result of the stress of birth and through the activation of the sympathetic nervous system (12, 13). This hormonal environment is persistent throughout the suckling period and is thought to be related to the high fat and low carbohydrate content of the diet that is consumed by the newborn. What happens to circulating adiponectin levels following birth is not known.

Malonyl-CoA is a potent endogenous inhibitor of mitochondrial fatty acid oxidation. Malonyl-CoA is synthesized in the heart by ACC, the activity of which decreases after birth (14). ACC is phosphorylated and inactivated by AMPK (reviewed in Ref. 15), the expression and activity of which increases in the heart after birth (16). Malonyl-CoA is degraded in the heart by decarboxylation to acetyl-CoA by malonyl-CoA decarboxylase (MCD) (17), the activity of which also increases after birth (17,

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[1] The abbreviations used are: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; HMW, high molecular weight; MCD, malonyl-CoA decarboxylase.

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18). We have suggested that the simultaneous increase in MCD and decrease in ACC and malonyl-CoA contribute to the increase in fatty acid oxidation in the newborn period (16). Whether changes in adiponectin, gAd, or control of AMPK by adiponectin and/or gAd contribute to the increase in fatty acid oxidation has not been determined.

The objective of this study was to determine whether adiponectin and/or gAd are involved in the increase in fatty acid oxidation in the newborn heart. We also examined the interaction between insulin and gAd in the regulation of fatty acid oxidation in the newborn heart.

EXPERIMENTAL PROCEDURES

Animals—Hearts were obtained from 1- and 7-day-old New Zealand White rabbits, whereas plasma samples were obtained from 1- and 7-day-old and 6-week-old rabbits. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and all procedures on animals were approved by the Health Services Animal Welfare Policy Committee at the University of Alberta. On the morning of the experiments, 1- and 7-day-old rabbits were separated from the doe and anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. Six-week-old rabbits received an intravenous injection of 60 mg/kg sodium pentobarbital. When the rabbits totally lacked sensation, the thoracic cavity of 1-day-old rabbits was opened, and the heart was quickly excised and placed in ice-cold Krebs-Henseleit solution. Blood was collected from 1- and 7-day-old and 6-week-old rabbits, and plasma was separated by centrifugation (10,000 $\times$ *g* for 10 min).

Western Blot Analysis of Adiponectin in Plasma—Plasma samples of 1- and 7-day-old and 6-week-old rabbits were subjected to SDS-PAGE by using the method of Laemmli (19). Following transfer onto nitrocellulose, bands were probed with a polyclonal antibody directed against adiponectin (Chemicon International, 1:2000 dilution in Tris-buffered saline with 0.05% Tween 20 in 1% non-fat milk). After reaction with a secondary antibody (anti-mouse IgG-horseradish peroxidase), the blots were visualized with chemiluminescent detection by using an ECL Western blotting detection kit. Adiponectin concentrations in samples were then calculated from a linear standard curve generated by increasing amounts of mouse recombinant adiponectin on the same gel.

Recombinant Protein Production and Protein Characterization—Recombinant adiponectin was produced by following the protocol described in Fruebis *et al.* (3) except that cDNA was cloned in pET-30a (Novagen), and a chelating Sepharose fast flow column was used to isolate the N-terminal His_6-tagged fusion protein from the lysed bacterial pellet. To detect the full-length adiponectin, the end product was separated by SDS-PAGE and transferred onto nitrocellulose paper by standard procedures. Horseradish peroxidase-conjugated anti-His tag antibody (Santa Cruz Biotechnology) and mouse anti-adiponectin antibody raised against the globular head domain (Chemicon) followed by horseradish peroxidase-conjugated anti-mouse IgG ((Santa Cruz Biotechnology) were used to visualize the proteins.

Sucrose Gradient Separation of Adiponectin Multimers—Adiponectin multimers and protein standards (Novagen) were separated on a 5–20% sucrose gradient, as described previously (20). Gradients were spun in 3.2-ml centrifuge tubes at 259,000 $\times$ *g* for 259 min in an SW55Ti ultracentrifuge rotor (Beckman). Sequentially, 160-μl fractions were removed and subjected to Western blot analysis with an anti-His_6 horseradish peroxidase-labeled antibody (Santa Cruz Biotechnology).

Heart Perfusions—Isolated hearts obtained from 1-day-old rabbits were perfused in Langendorff mode at a coronary perfusion pressure of 20 mm Hg. Hearts from 7-day-old rabbits were perfused by using the working heart model subjected to a preload pressure of 7.5 mm Hg and an aortic afterload of 30 mm Hg. The hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose, 0.4 mM [1-^{14}C]palmitate (Amersham Biosciences), or [9,10-^{3}H]palmitate (PerkinElmer Life Sciences), 3% bovine serum albumin, with or without 100 microunits/ml insulin (Gibco, crystalline bovine). Fatty acid oxidation rates were measured by quantitative collection of $^{14}CO_2$ or $^{3}H_2O$ every 10 min, as described previously (21, 22).

Isolated working hearts from 7-day-old rabbits were perfused as described previously (20). Hearts were perfused with Krebs-Henseleit solution containing 0.4 mM [1-^{14}C]palmitate, 3% bovine serum albumin, 11 mM glucose, 100 microunits/ml insulin, and fatty acid oxidation rates determined as described (19, 21).

Tissue Preparation for AMPK and ACC Activity Assays—Approximately 50 mg of frozen ventricular tissue from previously perfused hearts was homogenized using a Polytron® homogenizer for 30 s at 4 °C in 400 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM sodium fluoride, 5 mM sodium pyrophosphate, 10% (w/v) glycerol, and 0.1% (v/v) of mammalian protease inhibitor mixture (Sigma). Following centrifugation at 9000 $\times$ *g* for 5 min at 4 °C, protein content was measured using the Bradford method (23). For the AMPK activity measurements, 0.1% (v/v) Triton X-100 was included in the protein samples.

AMPK Activity Assay—AMPK activity was assayed in whole tissue homogenates by following the incorporation of [γ-^{32}P]ATP into the synthetic peptide AMARA. The assay was performed in a 25-μl total volume containing 40 mM HEPES-NaOH (pH 7.0), 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 200 μM AMARA peptide, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, 200 μM ATP (containing [γ-^{32}P]ATP). The assay was performed in the absence or presence of 200 μM 5′-AMP at 30 °C for 5 min. The reaction was initiated by the addition of 200 μM ATP and 5 mM $MgCl_2$. At the end of reaction, 15-μl aliquots were removed and spotted on a 1 $\times$ 1-cm square of phosphocellulose paper (P81®, Whatman), which were then placed into 150 mM phosphoric acid. These papers were washed four times for 10 min with 150 mM phosphoric acid and then once with acetone. The radioactivity of the dried papers was determined by using standard liquid scintillation procedures.

Western Blot Analysis of Ser-79-phosphorylated ACC—Heart homogenates (30 μg) were subjected to SDS-PAGE by using standard methods. Following the gel electrophoresis, the protein bands were transferred to nitrocellulose. Membranes were then probed with rabbit polyclonal antibody against phosphorylated (Ser-79) ACC (Upstate Biotechnology, Inc.). Secondary peroxidase-conjugated goat anti-rabbit IgG was used to visualize phospho-ACC. Chemiluminescent detection was performed on the membranes using an ECL Western blot detection kit.

ACC Activity Assay—ACC activity was measured in heart homogenates using the $^{14}CO_2$ fixation method (24, 25). The assay mixture contained 60.6 mM Tris acetate (pH 7.5), 1 mg/ml bovine serum albumin, 1.3 μM 2-mercaptoethanol, 2.1 mM ATP, 1.1 mM acetyl-CoA, 5 mM magnesium acetate, 18.2 mM $NaHCO_3$ (containing $NaH^{14}CO_3$). Following a 2-min incubation at 37 °C, the reaction was stopped by adding 25 μl of 10% perchloric acid. Following centrifugation at 2000 $\times$ *g* for 20 min, radioactivity of the supernatant was determined by using the standard liquid scintillation counting procedures.

Determination of CoA Esters—CoA esters were determined in 6% perchloric acid extracts from frozen heart tissues using a modified high pressure liquid chromatography procedure, as described previously (14).

Statistical Analysis—Data are expressed as mean $\pm$ S.E. The unpaired Student's *t* test was used to determine statistical significance between two separate group means. One-way analysis of variance followed by Tukey-Kramer was used when two subsequent measurements in one group were compared with two subsequent measurements in the other. A value of $p < 0.05$ was regarded as significant.

RESULTS

Plasma Levels of Adiponectin in 1- and 7-Day-old and 6-Week-old Hearts—Fig. 1 shows the relative amounts of adiponectin in plasma samples collected from 1- and 7-day-old rabbits. Fig. 1*A* is a representative Western blot, and Fig. 1*B* is the densitometric analysis of Fig. 1*A*. Plasma levels of adiponectin increased between 1 and 7 days of age. The antibody that we used (Chemicon) in this experiment was raised against the gAd domain of the peptide. We also observed a signal that corresponds to an ~16-kDa protein (gAd) on the immunoblots (data not shown). However, the signal was very weak and did not allow us to determine the amount of gAd in the plasma.

Plasma levels of adiponectin were determined in the plasma of 1- and 7-day-old and 6-week-old rabbits using a quantitative Western blotting technique described under "Experimental Procedures." Plasma concentration of the full-length adiponectin was 1.2 $\pm$ 0.3 μg/ml in 1-day-old, 6.8 $\pm$ 1.8 μg/ml in 7-day-old, and 45 $\pm$ 5 μg/ml in 6-week-old rabbits (Table I).

Oligomerization State of Recombinant Adiponectin—The multimeric state of recombinant adiponectin was determined by sucrose gradient ultracentrifugation (Fig. 2). Recombinant adiponectin primarily formed hexamers and HMW species, whereas minute amounts of adiponectin existed as trimers. These results are consistent with the findings of Tsao *et al.* (26).

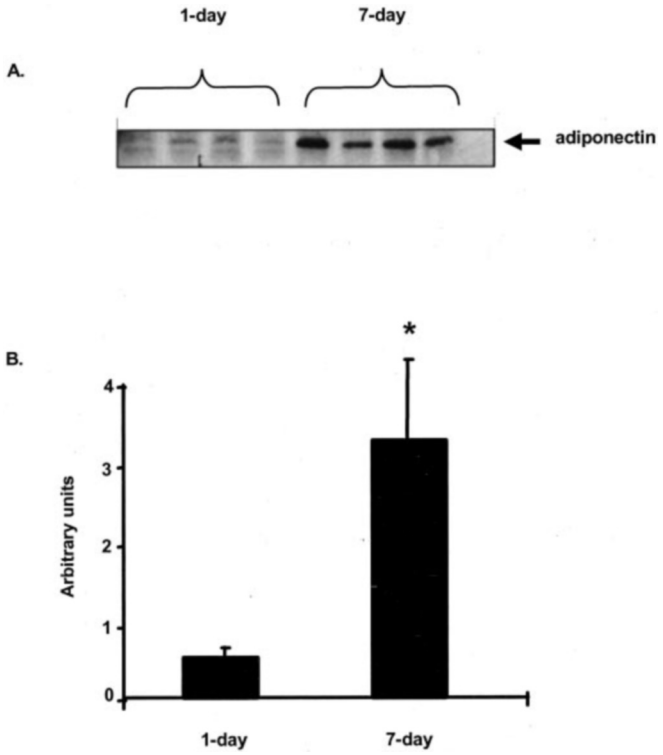


FIG. 1. **Adiponectin levels in plasma samples from 1- and 7-day-old rabbits.** *A*, representative Western blot showing the relative amounts of the full-length form of adiponectin in plasma samples from 1- and 7-day-old hearts. Each lane represents an individual rabbit. *B*, densitometric analysis of the data in *A*. Values in the *bar graph* are mean ± S.E. of four plasma samples in each age group.

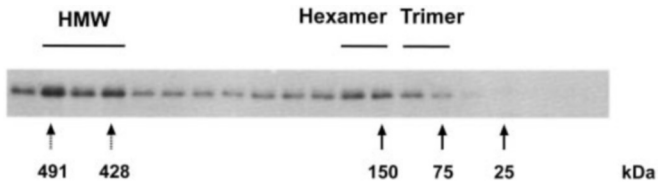


FIG. 2. **Oligomerization state of recombinant adiponectin.** Western blot of recombinant adiponectin (50 μg) separated by sucrose density ultracentrifugation. *Solid arrows* indicate the position in the gradient where standards of known molecular weights were separated. *Dashed arrows* denote an extrapolated molecular weight determined from the separation of known molecular weight standards.

TABLE I
The levels of adiponectin in newborn rabbit plasma

Plasma levels of full-length adiponectin were determined by using a quantitative Western blotting technique as described in detail under "Experimental Procedures." Values are the mean ± S.E. of four rabbits in each group.

Adiponectin		
1 day	7 days	6 weeks
	μg/ml	
1.2 ± 0.3	6.8 ± 1.8	45 ± 5

Fatty Acid Oxidation in 1-Day-old Hearts in the Presence of 10 Microunits/ml Insulin—Fatty acid oxidation rates were measured in 1-day-old hearts in the presence of 10 microunits/ml insulin. When added, adiponectin (10 μg/ml) was present throughout the entire 40-min perfusion period. Cumulative fatty acid oxidation in control and adiponectin-treated hearts were shown in Fig. 3*A*. Fig. 3*B* shows the average fatty acid oxidation rates throughout the 40-min perfusion in control and adiponectin-treated hearts. The recombinant full-length

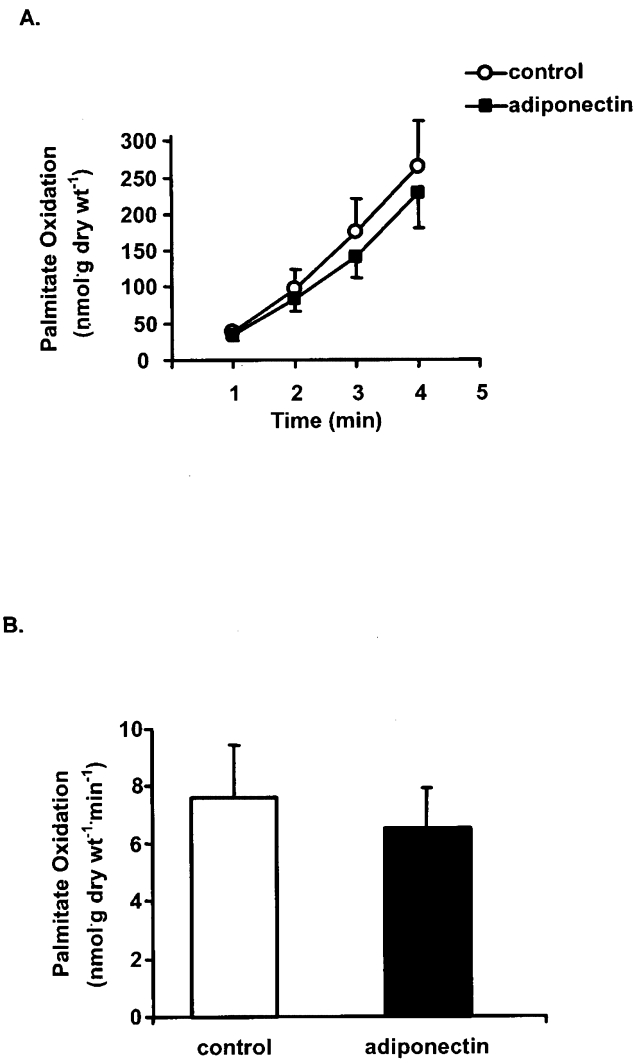


FIG. 3. **Effect of adiponectin on palmitate oxidation rates in 1-day-old hearts perfused in the presence of 10 microunits/ml insulin.** Cumulative (*A*) and steady state (*B*) palmitate oxidation rates were measured in 1-day-old hearts using [1-^{14}C]palmitate as described under "Experimental Procedures." Adiponectin (10 μg/ml) was added at the beginning of the 40-min perfusion protocol. Values are mean ± S.E. of six control and seven adiponectin-treated hearts.

adiponectin preparation at this concentration did not affect fatty acid oxidation rates in 1-day-old rabbit hearts.

Fatty Acid Oxidation in 1-Day-old Hearts in the Absence of Insulin—Fig. 4 shows fatty acid oxidation rates in 1-day-old hearts perfused in the absence of insulin. In this series of experiments, gAd (1.5 μg/ml) was added 30 min into the perfusion (Fig. 4*A*). gAd resulted in a rapid increase in fatty acid oxidation compared with control hearts (Fig. 4*A*). Comparison of the last 10 min of the perfusion with the initial 30-min period (Fig. 4*B*) showed that gAd more than doubled fatty acid oxidation rates.

AMPK Activity of Control and gAd-treated Hearts in the Absence of Insulin—To determine whether the AMPK pathway was involved in the effects of gAd on fatty acid oxidation, AMPK activity was measured at the end of the 40-min perfusion period in control and gAd-treated hearts. AMPK activity was similar between control and gAd-treated hearts (Fig. 5).

Phosphorylation and Activity of ACC in Control and gAd-treated Hearts—To verify that the increase in fatty acid oxidation was indeed independent of AMPK pathway, the phosphorylation status of Ser-79 and the activity of ACC were examined (Ser-79 is the AMPK phosphorylation site on ACC).

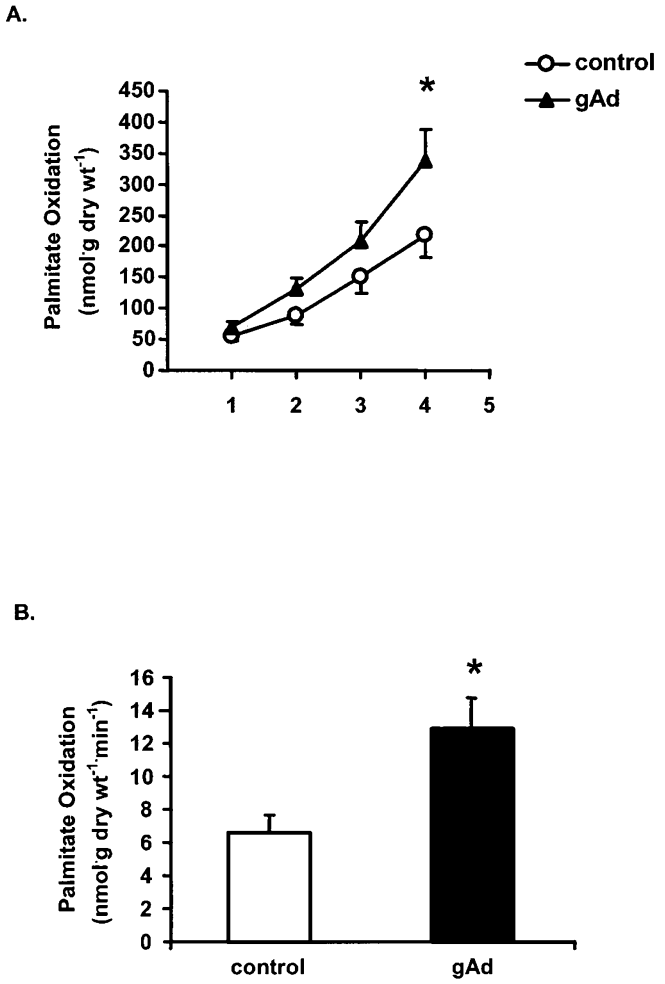


FIG. 4. **Effects of gAd on palmitate oxidation rates in 1-day-old hearts perfused in the absence of insulin.** Cumulative (*A*) and steady state (*B*) palmitate oxidation rates were measured in 1-day-old hearts using [1-^{14}C]palmitate, as described under "Experimental Procedures." The total length of perfusion was 40 min. gAd (1.5 μg/ml) was present during the last 10 min of the perfusion protocol. Values are mean ± S.E. of seven hearts in each group. * means significantly different from palmitate oxidation rate in the control group.

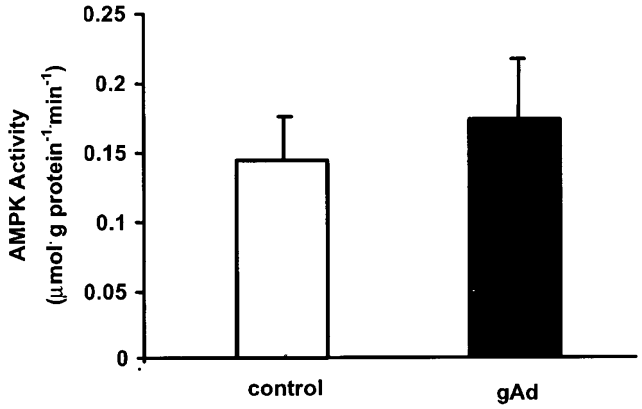


FIG. 5. **Effect of gAd on AMPK activity in 1-day-old rabbit hearts.** AMPK activity was measured in 1-day-old hearts perfused with (gAd) or without (control) gAd in the last 10 min of the perfusion protocol as described under "Experimental Procedures." The perfusate did not contain insulin. Values are mean ± S.E. of seven hearts in each group.

Fig. 6*A* is a Western blot showing Ser-79-phosphorylated ACC. As reported earlier by our group (13), rabbit heart expresses both isoforms of ACC (ACC265 and ACC280) to an equal extent. gAd treatment did not alter the extent of ACC Ser-79 phosphorylation (Fig. 6, *A* and *B*) or the activity of ACC (Fig. 6*C*).

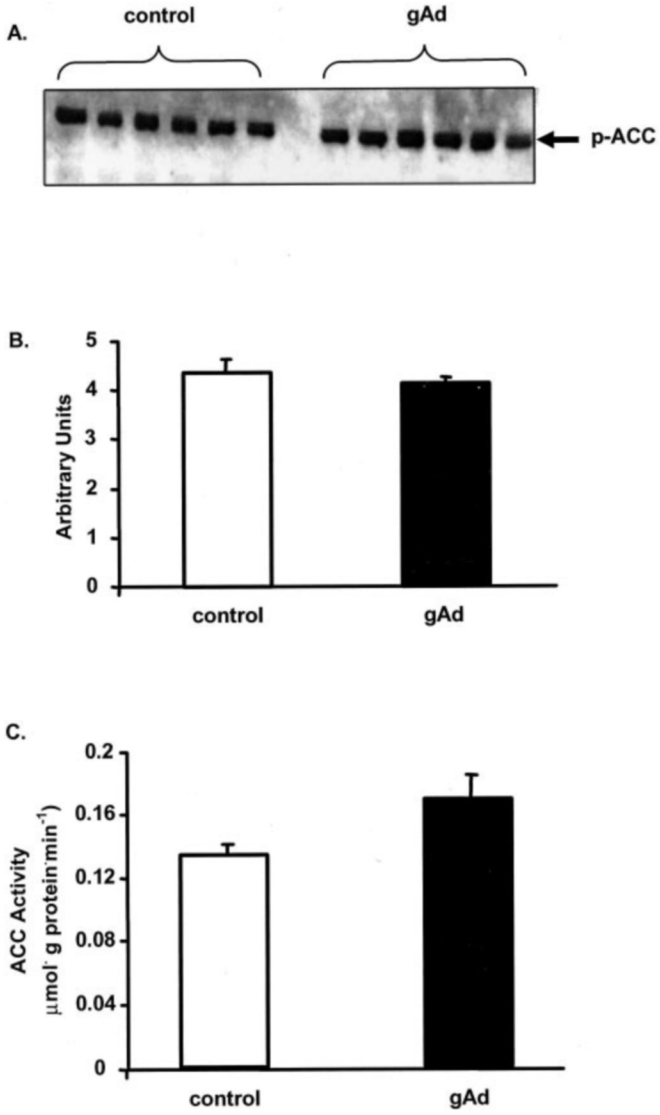


FIG. 6. **Effects of gAd on Ser-79 phosphorylation and ACC activity in 1-day-old rabbit hearts.** *A*, immunoblot analysis of Ser-79-phosphorylated ACC in 1-day-old hearts perfused with (gAd) or without (control) gAd. The perfusate did not contain insulin. Each lane represents one heart. *B*, densitometric analysis of the immunoblot shown in Fig. 5*A*. Values in the *bar graph* are mean ± S.E. *C,* ACC activity was measured in 1-day-old hearts perfused with (gAd) or without (control) gAd.

Malonyl-CoA and Acetyl-CoA Levels—The presence of gAd did not change the amount of malonyl-CoA (Table II). The amount of acetyl-CoA tended to increase; however, this increase did not reach statistical significance (Table II).

Fatty Acid Oxidation in 1-Day-old Hearts Perfused in the Presence of Insulin—To determine whether insulin altered the gAd-stimulated increase in fatty acid oxidation, fatty acid oxidation rates were measured in the presence of 100 microunits/ml insulin, with or without 1.5 μg/ml gAd. In the first set, gAd was added at the beginning of the perfusion protocol. In the presence of 100 microunits/ml insulin, fatty acid oxidation rates were similar in control and gAd-treated hearts throughout the 40-min perfusion (Fig. 7*A*).

We extended our examination in these hearts further, and we added gAd (1.5 μg/ml) during the last 10 min of the perfusion to provide similar conditions as in the non-insulin perfu-

TABLE II
Malonyl-CoA and acetyl-CoA content in 1-day-old hearts

Malonyl-CoA and acetyl-CoA levels were measured at the end of the 40-min perfusion in control hearts and in hearts treated with 1.5 μg/ml gAd during the last 10 min of the perfusion protocol. Values are the mean ± S.E. of seven hearts in each group.

	Control	gAd
	pmol·mg dry weight^{-1}	
Malonyl-CoA	2.3 ± 0.2	2.3 ± 0.4
Acetyl-CoA	85.8 ± 3.9	121.6 ± 26.7

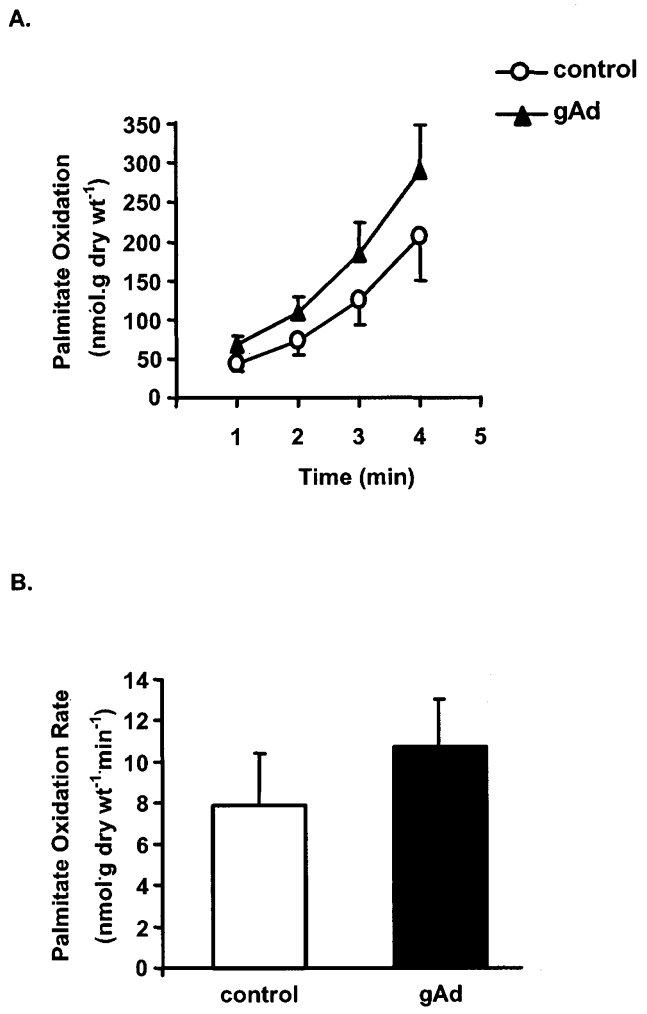


FIG. 7. **Effects of gAd on palmitate oxidation rates in 1-day-old hearts perfused in the presence of insulin.** Cumulative (*A*) and steady state (*B*) palmitate oxidation rates were measured in 1-day-old hearts in the presence of (100 microunits/ml) insulin using [1-^{14}C]palmitate as described under "Experimental Procedures." gAd (1.5 μg/ml) was added during the last 10 min of the perfusion protocol. Values are mean ± S.E. of 5–7 hearts in all groups.

sion series. Again, gAd had no effect on fatty acid oxidation in the presence of 100 microunits/ml insulin (Fig. 7*B*).

Fatty Acid Oxidation in 7-Day-old Hearts Perfused in the Absence of Insulin—Table III shows the effects of gAd on fatty acid oxidation rates and AMPK activity in isolated working hearts from 7-day-old rabbits. Based on the full-length adiponectin concentrations, we performed isolated heart perfusions using 10 μg/ml full-length adiponectin. Plasma concentration of the full-length adiponectin was 6.8 ± 1.8 μg/ml in 7-day-old rabbits. By using full-length adiponectin at this concentration, we created a hormonal environment that corresponds to 7-day-old plasma, and we investigated whether adiponectin stimulated fatty acid oxidation. As expected, fatty acid oxidation rates were substantially higher in 7- than 1-day-old hearts (5-fold higher). However, gAd did not have any significant effect on fatty acid oxidation rates. Similar to what was observed in 1-day-old hearts, gAd also did not have any effects on AMPK activity in the 7-day-old hearts.

TABLE III
Palmitate oxidation rates and AMPK activities in 7-day-old rabbits

Steady state palmitate oxidation rates were measured in 7-day-old hearts using [9,10-^{3}H]palmitate as described under "Experimental Procedures." gAd (1.5 μg/ml) was added at the beginning of the 40-min perfusion protocol. Values are the mean ± S.E. of 5 control and 13 gAd-treated hearts. AMPK activity was determined in whole tissue homogenates of the hearts that were frozen at the end of the 40-min perfusion.

Palmitate oxidation	
Control	gAd
nmol·g dry weight^{-1}·*min*$^{-1}$	
414 ± 52	455 ± 33

To determine whether AMPK activation was capable of stimulating fatty acid oxidation in 1-day-old hearts, we perfused hearts with 200 μM 5-aminoimidazole-4-carboxamide riboside (paced at 250 beats/min, because 5-aminoimidazole-4-carboxamide riboside is a negative chronotropic agent). This resulted in an increase in fatty acid oxidation rates from 759 ± 194 to 1660 ± 280 nmol/g dry weight/min (n = 7 and 6 hearts, respectively) while increasing AMPK activity to 165% of control values. As a result, it was clear that AMPK activation can increase fatty acid oxidation rates in the newborn hearts.

DISCUSSION

The newborn period is characterized by dramatic alterations in energy substrate supply, with fatty acid oxidation becoming a predominant source of energy following birth (7). Alterations in hormone concentrations play a role in this process, with a decrease in insulin and an increase in glucagon contributing to the increase in fatty acid oxidation (16). In this study we show that plasma levels of adiponectin increase following birth and may contribute to the postnatal increase in fatty acid oxidation. In particular, a truncated form of adiponectin, gAd, was shown to increase acutely fatty acid oxidation in 1-day-old rabbit hearts. In contrast, full-length adiponectin did not have any effect on cardiac fatty acid oxidation rates. In previous studies, we have shown that a decrease in malonyl-CoA levels contributes to the increase in fatty acid oxidation in the newborn heart (14). Of interest is that this effect of gAd on fatty acid oxidation occurred independent of changes in malonyl-CoA levels or AMPK and ACC control of malonyl-CoA levels.

Adiponectin is a peptide hormone and is secreted exclusively by the differentiated adipocytes (1). Although the initial signal to increase the plasma levels of adiponectin is currently not known, a decrease in insulin may be the triggering event that increases adiponectin gene expression. In the immediate newborn period, insulin levels dramatically decrease (from ~100 to less than 10 microunits/ml) (7). Insulin decreases adiponectin mRNA in a 3T3-L1 cell line in a dose-dependent fashion (27). Whether the suppressant effect of insulin on mRNA is translated to the protein was not addressed in that study. In this study, we show that plasma adiponectin levels dramatically increase following birth, at the same time insulin levels decrease (7).

Recently, Combs *et al.* (28) also showed that levels of adiponectin increase in postnatal life in mice. The time course of the maturation of mouse heart is not known. However, we have shown previously that in rabbit heart fatty acid oxidation increases within the first week of the postnatal life. This is

consistent with the rise in adiponectin between 1 and 7 days following birth (Fig. 1).

Adiponectin is present in serum at high concentrations and exists as several molecular weight forms in the plasma (1). This raises questions as to the precise composition in the plasma and, more importantly, what is the active form of this protein. Recently, Tsao *et al.* (26) reported that the largest species (apparent molecular mass, 410 kDa) produced by *Escherichia coli* was an adiponectin hexamer, a finding we also observed in our study (Fig. 2). However, adiponectin also forms high molecular weight (HMW) species of apparent molecular mass of ~629 kDa in the plasma of both mice and human (26, 28). The biological activity of these isoforms depends on the oligomer formation. Pajvani *et al.* (20) reported that trimeric adiponectin was the most potent isoform in suppressing glucose production in the hepatocytes. Recently, Tomas *et al.* (5) reported that the hexameric form of adiponectin did not activate AMPK in rat extensor digitorum longus muscle. Kobayashi *et al.* (29) also showed that the trimeric form of adiponectin can activate AMPK in human umbilical vein endothelial cells but not the hexameric and HMV forms. As a result, it is possible that the lack of effect of adiponectin on AMPK in our hearts could be due to the lack of trimeric adiponectin in our preparation. In contrast to the full-length adiponectin, the globular domain of adiponectin, which exists as a single trimeric species in our preparation (28), was able to stimulate fatty acid oxidation in the heart.

During the immediate postnatal period, plasma glucagon increases and plasma insulin falls and remains in a low range concentration in newborns of different species (7). It has been suggested that the neonatal increase in plasma glucagon and the fall in plasma insulin could be related to the stress of birth through an activation of the sympathetic nervous system (12, 31). Catecholamines may trigger changes in plasma insulin and glucagon during the immediate newborn period, because they are potent stimuli of glucagon release and inhibitor of insulin release (32, 33). Previously, we reported (16) that both expression and activity of AMPK increase between 1 and 7 days in newborn rabbit hearts. We also showed that insulin inhibits AMPK activity in the heart (34), suggesting that a decrease in insulin levels *in vivo* may stimulate AMPK activity following birth (16). As a result, insulin may have a dual role following birth, directly altering malonyl-CoA and fatty acid oxidation in the newborn heart and indirectly altering hormone levels such as adiponectin.

A number of studies (4–6) recently showed that adiponectin/gAd increases fatty acid oxidation by stimulating AMPK. AMPK phosphorylates and inactivates ACC, the enzyme that is responsible for malonyl-CoA synthesis. Because gAd stimulated fatty acid oxidation in the newborn heart, we investigated whether the AMPK-ACC-malonyl-CoA pathway was involved in the gAd-induced increase in fatty acid oxidation. We did not observe a change in phosphorylation status of ACC, activity of ACC, or AMPK activity in 1-day-old hearts. We also did not observe any effect of gAd on AMPK activity in 7-day-old hearts. Malonyl-CoA levels were also similar between 1-day-old control and gAd-treated hearts. As a result, our data suggest that the increase in fatty acid oxidation induced by gAd in 1-day-old hearts is independent of AMPK activation

The mechanism by which gAd increases fatty acid oxidation in the newborn heart remains to be determined. Of interest is that insulin (100 microunits/ml) could overcome the gAd stimulation of fatty acid oxidation. Adiponectin at a concentration of 10 μg/ml had no effect on fatty acid oxidation. It is attractive to hypothesize that gAd rather than adiponectin stimulates fatty acid oxidation by binding to its high affinity receptor in the heart as suggested by Yamauchi *et al.* (6). However, the lack of effect of the full-length adiponectin could also be related to the fact that a low level of insulin was present (10 microunits/ml) in these experiments. In this study, we did not perform any perfusions with adiponectin in the complete absence of insulin. It is possible that adiponectin may stimulate fatty acid oxidation in the complete absence of insulin. However, unlike gAd, we also did not observe an increase in fatty acid oxidation in isolated working adult mouse hearts with the full-length adiponectin.[2]

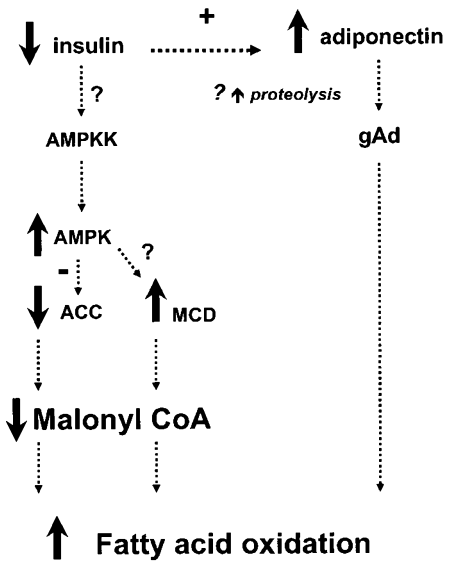


FIG. 8. **Proposed pathway by which fatty acid oxidation increases in the newborn heart.** A decrease in insulin relieves the inhibition of AMPK resulting in an increase in AMPK activity. The increase in both AMPK expression and activity results in a decrease in ACC activity. MCD expression and activity also increases. A decrease in ACC activity and increase in MCD activity in the newborn period results in a dramatic drop in malonyl-CoA. A decrease in circulating insulin triggers adiponectin/gAd release from the adipocytes. Adiponectin is cleaved to gAd which stimulates fatty acid oxidation. This occurs via an AMPK-ACC independent pathway. *AMPKK,* AMPK kinase.

In our initial perfusions, we included insulin (10 microunits/ml) to the perfusate. Addition of full-length adiponectin at a concentration similar to that seen in the plasma of 7-day-old rabbits did not affect the fatty acid oxidation rates in the presence of this concentration of insulin. The levels of plasma adiponectin that we report in this study (Table I) are based on the trimeric structure that has a molecular mass of 90 kDa (3× 30 kDa). When selecting the concentration for treatment, we based our calculations on the trimeric form of adiponectin. We use a bacterial expression system (*E. coli*) to prepare the full-length adiponectin. The protein mixture prepared by this system has been shown to contain the trimeric and hexameric forms of adiponectin but not the HMW form (26, 30). Therefore, it cannot be ruled out that the lack of effect with adiponectin treatment in our perfusions was because of the absence of the HMW form of adiponectin in our preparation.

In conclusion, our results suggest that a decrease in plasma insulin and an increase in plasma gAd are involved in the increase of cardiac fatty acid oxidation in the immediate newborn period. The increase in fatty acid oxidation is not mediated by the AMPK-ACC-malonyl-CoA pathway. We propose the scheme shown in Fig. 8. The identification of the signal that results in the cleavage of the full-length adiponectin to gAd and the reduction of the HMW pool of adiponectin to lower molecular forms is required to map the intracellular events that

[2] R. Kozak, J. Altarejos, and G. Lopaschuk, unpublished observations.

result in the maturation of fatty acid oxidation in the newborn period.

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