

## Links between De Novo Fatty Acid Synthesis and Leptin Secretion in Bovine Adipocytes

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**ABSTRACT.** Leptin secretion by adipose tissue is involved in many physiological control systems, including those that determine growth, development, body composition, milk production, and reproductive function. In the adipocyte of monogastric animals, malonyl CoA (coenzyme A) seems to link the flux of energy substrates to the control of leptin production. In this study, we tested this for ruminants by examining the effect of cerulenin, an inhibitor of de novo fatty acid synthesis at the step from malonyl CoA to palmitate, on leptin production by cultured bovine adipocytes derived from intermuscular fat. Purified preadipocytes were obtained by the ceiling culture method, and adipogenic media were used to induce their differentiation into adipocytes. We found that leptin concentrations increased significantly with time in culture, and with increases in glucose concentration. Addition of 2-deoxy-D-glucose to the medium, a competitive inhibitor of glucose transport and metabolism, suppressed leptin secretion. In media with high glucose concentrations, cerulenin enhanced leptin secretion. We conclude that, as in monogastrics, malonyl CoA may play a key role in the control of leptin secretion in ruminants.

**KEY WORDS:** 2-Deoxy-D-glucose, cerulenin, malonyl CoA, ruminant.

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Adipose homeostasis mediated by leptin secretion seems to be involved in many physiological control systems, including those that determine growth, development, body composition, milk production, and reproductive function. The factors that regulate leptin synthesis by adipose tissue *in vivo* have been described for ruminants [4] but little is known of the cellular or biochemical processes involved. To date, most studies of intracellular control of leptin secretion by adipocytes have been done with non-ruminant models, such as laboratory rodents, in which it appears that there are control points in the pathways leading to the synthesis of fatty acids. Non-ruminants normally synthesize fatty acids by converting excess glucose to citrate in mitochondria via the tricarboxylic acid (TCA) cycle. The citrate then diffuses into the cytoplasm where it is split by adenosine triphosphate (ATP) citrate lyase to form malate and acetate. The acetate forms acetyl CoA (coenzyme A), which can then be condensed to form malonyl CoA and, ultimately, palmitate. Recently, it has been shown that when cerulenin is added to culture medium for rodent adipocytes, de novo synthesis of fatty acids is inhibited, malonyl CoA accumulates, and, at the same time, leptin secretion is stimulated [25]. This suggests that malonyl CoA controls leptin secretion.

Would the same observation apply in cattle given that ruminants tend to restrict the flow of carbon atoms to fatty acids from glucose? Under normal circumstances, ruminants do not synthesize fatty acids from glucose because, in contrast to non-ruminants, glucose is the minor energy-substrate form absorbed from their digestive tract. In addition, ATP citrate lyase is either absent or present in such low con-

centrations that the flow of carbon from glucose is severely restricted [2, 5, 16, 24]. Ruminants synthesize all the glucose that they need via gluconeogenesis from amino acids or from the volatile fatty acid, propionate, and the main precursors for synthesis of fatty acids are the volatile fatty acids themselves, acetate and butyrate. These short-chain fatty acids can be used to form acetyl CoA in the cytoplasm, and this leads to production of palmitate by the same set of reactions as in non-ruminants. In summary, the main difference between ruminants and non-ruminants in *de novo* synthesis of fatty acids, and thus perhaps the control of leptin secretion, is the source of the cytoplasmic precursor, acetate. However, glucose is also the precursor for lipogenesis in ruminants. Indeed, acetate provides 70–80% and 10–25% of the acetyl CoA for lipogenesis in subcutaneous adipose and intramuscular adipose tissues, respectively. Conversely, glucose provides 1–10% and 50–70% of the acetyl CoA in subcutaneous and intramuscular adipose tissues, respectively [26, 27]. Furthermore, during culture of ovine adipose tissue for 3 days, the rate of fatty acid synthesis from acetate declined, whereas the rate of fatty acid synthesis from glucose continued to increase [31]. Since the condensation reactions of acetyl CoA to palmitate are the same in ruminants and non-ruminants, we suggest that the control point for leptin is the same in ruminants and non-ruminants.

Due to the lack of an appropriate *in vitro* model, our knowledge about leptin secretion by bovine adipocytes has been limited. Recently, in a study focussed on the control of marbling in Japanese black beef cattle, we detected leptin messenger ribonucleic acid (mRNA) in cultured adipocytes

derived from intermuscular fat (K. Aikawa, unpublished). We therefore tested whether these adipocytes utilize intracytoplasmic malonyl CoA to control leptin secretion by combining the response to cerulenin with a sensitive radioimmunoassay (RIA) based on recombinant bovine leptin [3].

## MATERIALS AND METHODS

*Ceiling culture for preparing preadipocytes and induction of differentiation into adipocytes:* Bovine preadipocytes were obtained by the 'ceiling culture' method [21, 28, 34]. The basic steps involved were: 1) isolation of adipocytes from other cells based on their unique ability to float in culture media; 2) firm adhesion of the isolated adipocytes to the top inner surface ('ceiling') of a flask filled with culture media; and 3) formation of colonies of preadipocytes from the adipocytes on the ceiling during subsequent culture for more than 10 days at 37°C under an atmosphere of 95% air and 5% CO<sub>2</sub>. Using this method, we prepared preadipocytes from intermuscular fat from the 4th to 5th rib sections of Japanese black beef cattle (26 months old). These cells formed a subconfluent monolayer in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, U.S.A.) containing 100 U/ml penicillin G, 100 µg/ml streptomycin sulphate, and fetal calf serum (FCS; Dainippon Pharmaceutical, Osaka, Japan). We utilized 20% FCS because our preliminary experiment confirmed that using serum concentration of more than 10% was the most desirable concentration for the growth of bovine preadipocytes, as reported previously [1]. We then treated the cells with trypsin (Gibco), suspended them in the culture media and stored them at -80°C in cryoprotectant (Cell Banker 2, NihonZenyaku, Fukushima, Japan) in cryotubes at a density of about  $4.8 \times 10^5$  cells/tube.

Thawed preadipocytes were gently suspended in 10 ml culture media, centrifuged, and then resuspended in 20 ml media. About  $1.2 \times 10^5$  cells were incubated in each 250 ml flask at 37°C under an atmosphere of 95% air and 5% CO<sub>2</sub>. Subconfluent preadipocytes were then trypsinized and sus-

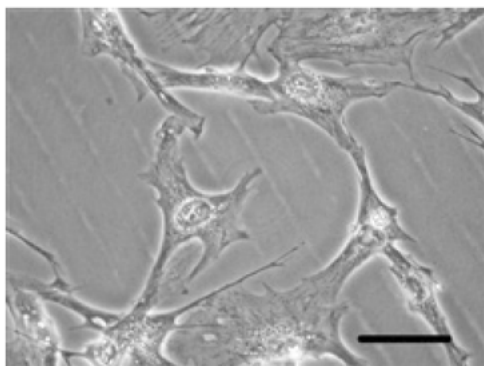
pended in culture media at a density of about  $3.2 \times 10^4$  cells/ml. Then, 1 ml of the suspension was incubated in each of 12 wells in flat bottom plates until they again formed confluent monolayers.

Differentiation of preadipocytes (fibroblast-like shape; Fig. 1a) into adipocytes was induced by replacement of the standard culture medium with 1 ml adipogenic culture medium containing 5 µg/ml insulin (Wako, Osaka, Japan), 1.5 µM of T174 (Tanabe Seiyaku, Osaka, Japan), 0.5 mM isobutylmethylxanthine (Wako), 0.25 µM dexamethasone (Wako), and 20% FCS. T174 is a synthetic ligand for Peroxisome Proliferator-Activated Receptor-gamma (PPAR-gamma) that reportedly has a strong stimulatory effect on adipose differentiation [23, 30]. Both isobutylmethylxanthine and dexamethasone have a role in stimulating genes to produce PPAR-gamma during the very early stages of differentiation from preadipocytes to adipocytes [6, 29, 33]. After incubation for 4 days, the medium was replaced with a second adipogenic medium containing 5 µg/ml insulin, 1.5 µM T174, and 20% FCS, and culture was continued for 4 more days. After differentiation, all cells appeared to be adipocytes and contained intracytoplasmic lipid droplets that could readily be observed microscopically after the Oil Red Zero staining (a round, fat shape due to the reserved intracytoplasmic lipid; Fig. 1b). A preliminary experiment confirmed that leptin was secreted into the culture medium by the adipocytes, but not by the preadipocytes.

Immediately after differentiation, the adipogenic media in the wells were replaced with culture media containing the test compounds outlined below. In all experiments, hour 0 was defined as the time at which this was done.

*Cultured in the media with glucose, acetate, cerulenin, or 2-deoxy-D-glucose:* In order to determine appropriate conditions, two DMEM powders were used to prepare 20% FCS DMEM, one containing 3,600 mg/l glucose (#31600, Gibco) and one containing 800 mg/l glucose (#12800, Gibco). A 50:50 mixture of these two media containing 2,200 mg/l glucose was also prepared. It should be noted that values of around 570, 630, and 840 mg/l have been reported to be normal blood concentrations of glucose in

A



B

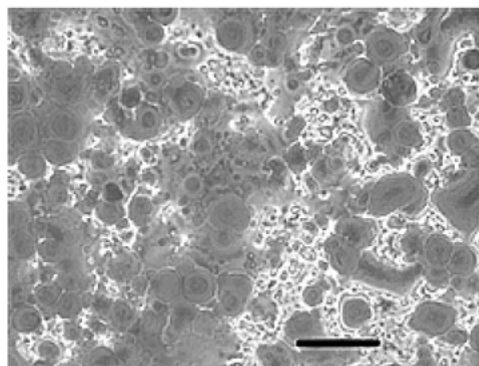


Fig. 1. Cultured preadipocytes observed using a phase contrast inverted microscope (A) and adipocytes after the Oil Red Zero staining (B). The length of black bars is 50 µm.

lactating dairy cows, prepartum dairy cows, and growing dairy heifers, respectively [8–10].

To define the changes in leptin production with the passage of time in culture, the adipogenic media in the wells were replaced immediately before each test with 2.5 ml/well of DMEM containing 3600 mg/l glucose, and then the adipocytes were cultured for 72 hr. During this period, 0.5 ml media was sampled from each well at 0, 24, 48, and 72 hr. With this sampling regime, the volume of culture media was reduced from 2.0 ml (0–24 hr) to 1.5 ml (24–48 hr) and then from 1.5 ml to 1.0 ml (48–72 hr), the minimum necessary to sustain reliable culture conditions. For all subsequent experiments except the additional study of the effects of 2-deoxy-D-glucose (2 DG), fewer samples were taken to minimize this effect. All samples were stored at  $-35^{\circ}\text{C}$  until assay.

In order to determine the appropriate concentrations of glucose in the media, the adipogenic media were replaced with 1 ml/well of DMEM containing either 800, 2,200 or 3,600 mg/l glucose, and then the adipocytes were cultured for 72 hr. At the end of 72 hr culture, a single sample of media was collected and assayed for leptin.

In order to evaluate the necessity of acetate in the culture media, 1 ml/well of DMEM (800 or 3,600 mg/l glucose) containing 0, 0.8, 2.5, 5, or 10 mM acetate was used for 72 hr culture, and then the media were collected and assayed for leptin. Among these concentrations, 2.5 mM has been reported to be a normal physiological concentration of acetate in bovine blood [14, 22]. We used maximum of 10 mM acetate because the pH falls below 7 with higher concentrations.

Subsequently, the effects of cerulenin on the increase in leptin concentration were determined during incubation by adding 2 ml/well of DMEM (3,600 mg/l glucose) along with 0, 10, 20, 40, or 80 mM cerulenin and culturing the adipocytes for 72 hr. Each well was sampled (1 ml) at 36 and 72 hr.

Additional experiments were conducted to evaluate the effect of a competitive inhibitor of glucose transport and metabolism, 2-deoxy-D-glucose (2DG), on the increase in leptin concentration during incubation.

The reasons for this additional study were as follows. First, we found that a higher glucose concentration also had a stimulatory effect on the cultured bovine adipocyte. Second, 2 DG reduces the leptin messenger RNA content of adipocytes and suppresses, but does not completely block, leptin secretion from cultured rat adipocytes and 3T3-L1 cells into culture media [18, 19]. For the additional study, 2.5 ml/well of DMEM containing 3600 mg/l glucose, with or without 500 mg/l 2DG, was used to culture the adipocytes for 72 hr. During this culture, 0.5 ml of the media was sampled from each well at 0, 24, 48, and 72 hr. We used a high dose of 2 DG first because it has been shown to exert a strong inhibitory effect on cultured rat adipocytes [18, 19]. We subsequently compared the responses to 0, 20, 100, and 500 mg/l of 2DG using 1 ml/well of DMEM containing 3,600 mg/l glucose for 72 hr culture. At the end of culture,

the media were collected and assayed for leptin. Furthermore, the inhibitory effects of 2DG (100 mg/l) on leptin secretion were also studied in media containing either 800, 2,200, or 3,600 mg/l glucose for 72 hr culture. At the end of culture, the media were collected and assayed for leptin.

**Assays:** Leptin was measured using our specific RIA based on recombinant bovine leptin [3]. All samples were processed in duplicate with a limit of detection of 0.07 ng/ml, and the intra-assay and interassay coefficients of variation were 6.8% and 5.8%.

**Statistical analyses:** The data were analyzed by Student's *t*-test or repeated measures or simple ANOVA with post hoc comparisons using Fisher's least significant difference. For the experiment in which we evaluated the effect of the interaction between acetate and glucose on leptin secretion, we used a two-factor ANOVA with post hoc comparisons using Fisher's least significant difference. All results are presented as means  $\pm$  SEM.

## RESULTS

In the experiment to define the time-course changes in leptin production during culture in DMEM containing 3,600 mg/l glucose, there was a progressive, logarithmic-type increase in the leptin concentrations of the samples of media as the time of incubation advanced ( $P < 0.01$ , Fig. 2).

In the second experiment to determine the appropriate concentration of glucose in the media, the leptin concentrations at 72 hr were higher ( $P < 0.01$ ) with 3,600 mg/l glucose DMEM than with 800 or 2,200 mg/l glucose DMEM (Fig. 3), and there was no significant difference between the values for 800 and 2,200 mg/l glucose DMEM.

In the experiment to evaluate the necessity of acetate in the culture media, leptin production was not significantly affected by acetate at any concentration tested nor was it

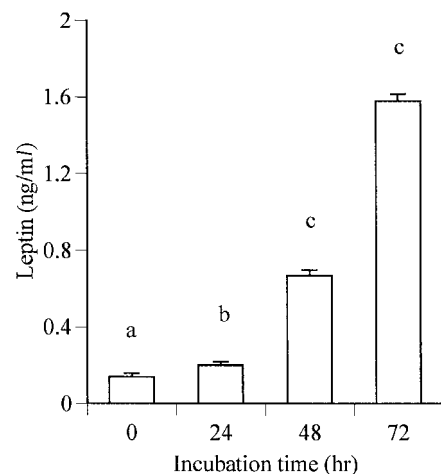


Fig. 2. Time-course changes in leptin concentration during culture in media containing 3,600 mg/l glucose. Values are means  $\pm$  SEM of 6 independent replicates. a, b  $P < 0.01$ , b, c  $P < 0.01$ .

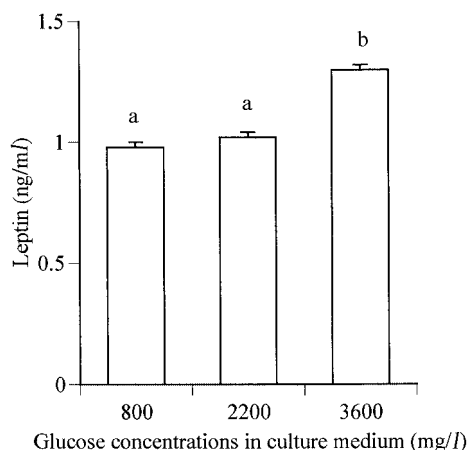


Fig. 3. Leptin concentrations after 72 hr culture of adipocytes in media containing 800, 2,200, or 3,600 mg/l glucose. Values are means  $\pm$  SEM of 6 independent experiments. <sup>a, b</sup>  $P < 0.01$ .

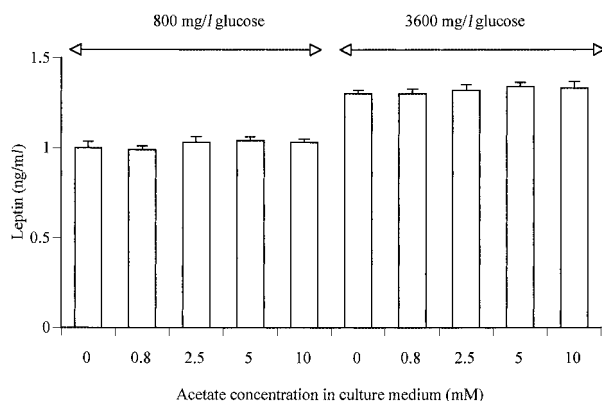


Fig. 4. Leptin concentrations after 72 hr culture of adipocytes in media containing 800 or 3,600 mg/l glucose and 0, 0.8, 2.5, 5, or 10 mM acetate. Values are means  $\pm$  SEM of 5 independent experiments.

affected by any interaction between acetate and glucose at glucose concentrations of 800 or 3,600 mg/l glucose (Fig. 4).

Then, we determined the main experiment design in order to evaluate the effects of cerulenin on the increase in leptin concentration during 72 hr culture using 3,600 mg/l glucose DMEM without acetate. In this experiment, all treatments with cerulenin increased leptin concentrations at 36 and 72 hr (Fig. 5).

In the supplementary experiments to evaluate the effect of 2 DG on the increase in leptin concentration during incubation, a reduction in the increase in leptin concentration was observed during culture with 500 mg/l 2DG (Fig. 6A). The reduction was observed for all three levels of 2DG treatment ( $P < 0.01$ ) and there was also evidence of a dose-response, with significant differences in 72-hr leptin values

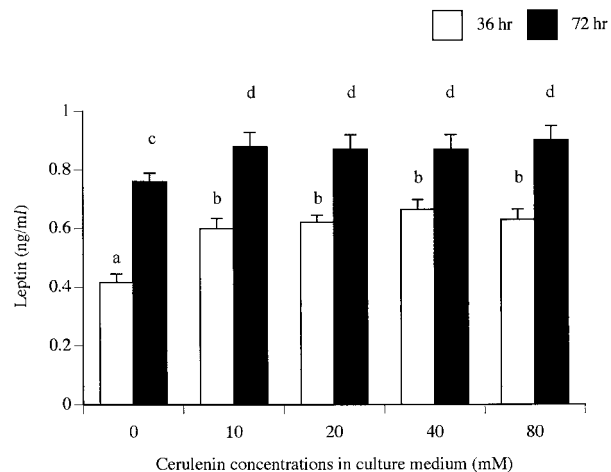


Fig. 5. Changes in leptin concentration during culture in media (3,600 mg/l glucose) containing 0, 10, 20, 40, or 80 mM cerulenin. Values are means  $\pm$  SEM of 5 independent experiments. <sup>a, b</sup>  $P < 0.01$ , <sup>c, d</sup>  $P < 0.05$ .

between 20, 100, and 500 mg/l 2DG (Fig. 6B). Inhibition of leptin secretion by 100 mg/l 2DG was also observed in media containing 800 or 2,200 mg/l glucose (Fig. 6C).

## DISCUSSION

The results of this study show that the control mechanism for leptin production for bovine adipocytes is linked to the pathways of *de novo* synthesis of fatty acids in much the same way as in the adipocytes of monogastric animals. Essentially, malonyl CoA is the major intracytoplasmic signal of the availability of energy, and, by controlling leptin secretion, it is a vital link in the 'adipostat' homeostatic system. This view is in agreement with observations from *in vivo* studies. In sheep, for example, intravenous infusion of propionate activates hepatic gluconeogenesis, increases the blood glucose concentration, and increases the levels of mRNA for both leptin and acetyl CoA carboxylase, an enzyme that synthesizes malonyl CoA from acetyl CoA [12]. Thus, the response of adipocytes to cerulenin, together with the responses to glucose, 2 DG, and acetate, are all consistent with the control pathway outlined in Fig. 7. Recent studies have revealed more details of the 'adipostat' homeostatic system in the mouse, and the following two additional pieces of information are available. Leptin stimulates fatty acid oxidation by activating 5'-adenylyc acid (AMP)-activated protein kinase, and this inhibits acetyl CoA carboxylase [17]. Malonyl CoA is the precursor of palmitate, and it also plays a role in reducing mitochondrial fatty acid oxidation [13]. Thus, leptin, 5'-AMP-activated protein kinase, acetyl CoA carboxylase, and malonyl CoA likely to comprise a system that controls lipogenesis, fatty acid oxidation, and energy homeostasis. Although further studies are necessary to confirm the details for ruminant adipocytes, a similar 'adipostat' homeostatic system may also exist in

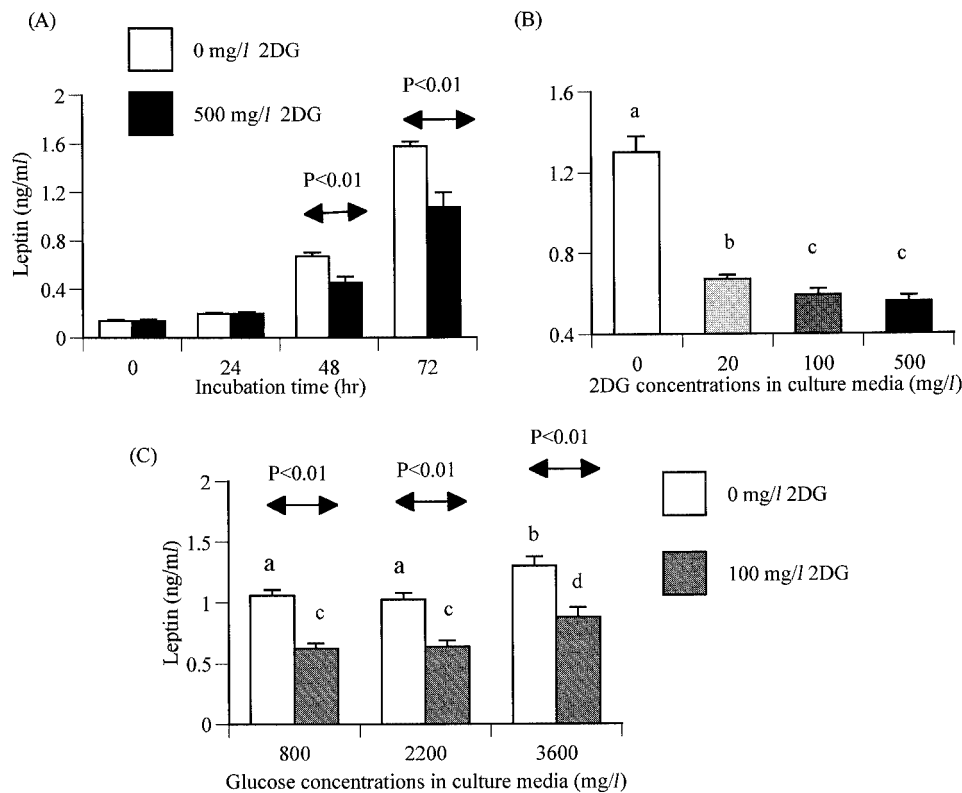


Fig. 6. (A) The effect of 500 mg/l 2DG on the increase in leptin concentration during culture in media containing 3,600 mg/l glucose; the black arrows indicate a significant effect for 2DG. Values are means  $\pm$  SEM of 6 independent experiments. (B) Relationship between the concentration of 2DG in the culture media (3,600 mg/l glucose) and leptin secretion from adipocytes. The bars indicate the means  $\pm$  SEM of 6 independent experiments. <sup>a, b</sup>  $P < 0.01$ , <sup>b, c</sup>  $P < 0.05$ . (C) Leptin concentrations after culture in media containing 800, 2,200, or 3,600 mg/l glucose with or without 100 mg/l 2DG. The black arrows indicated the significant effects of 2DG. The bars indicate the means  $\pm$  SEM of 6 independent experiments. <sup>a, b</sup>  $P < 0.01$ , <sup>c, d</sup>  $P < 0.01$ .

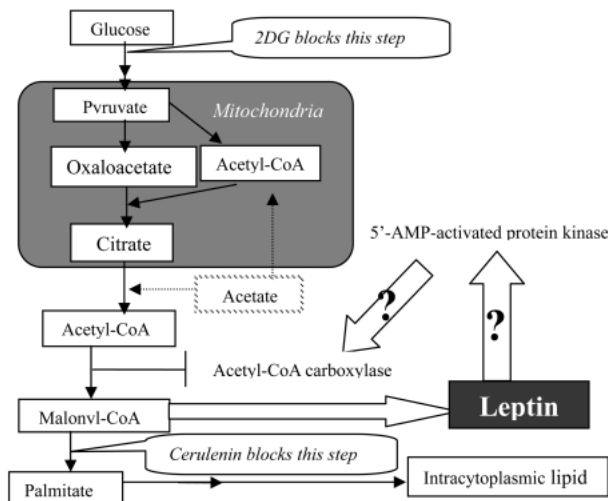


Fig. 7. Proposed intracellular mechanism for the regulation of leptin secretion in the cultured bovine adipocytes used in this study.

ruminant animals.

Both glucose and glucosamine induce leptin gene expression in 3T3-L1 cells [32, 38]. This is in agreement with studies using transgenic mice that overexpress the rate-limiting enzyme for hexosamine synthesis, which have higher circulating leptin concentrations than control mice [15]. Thus, monogastric animals may have another mechanism that controls leptin secretion by adipocytes based on the pathway via fructose-6-phosphate, which synthesizes glucosamine from glucose. This does not seem to apply to ruminants. In sheep, a 5-day infusion with glucosamine failed to affect the blood leptin concentration, whereas a 5-day glucose infusion was clearly stimulatory [20].

In bovine adipocytes, glucose stimulated and 2DG inhibited leptin secretion, although leptin production was similar with 800 and 2,200 mg/l glucose in the culture medium. This suggests that glucose is primarily a determinant of leptin secretion and that there is a threshold concentration above which leptin secretion is activated. However, it is important to remember that acetate is the main precursor for lipogenesis in ruminants and the effects of glucose on blood

leptin concentration may only be apparent under limited conditions. For example, a single intravenous injection of glucose does not stimulate leptin secretion in the shortterm (2 h) in sheep [11]. Our studies of intravenous infusions of glucose support this, although we did observe a robust postprandial increase in plasma leptin concentrations that could be maintained by glucose infusion [9]. On the other hand, a 5-day infusion with glucose increases blood leptin concentration in sheep [20]. To clarify the role of glucose *in vivo*, further studies are required.

We found that acetate (up to 10 mM) had no effect on leptin production. This same concentration was used by Yonezawa *et al.* They reported that it inhibited leptin mRNA expression in bovine mammary epithelial cells, a cell type that also secretes leptin [36], but that it stimulated expression in bovine anterior pituitary cells [35]. These disagreements may reflect the different cell types used, perhaps due to differences in resistance to the acetate dose or differences in the culture conditions that affected the relative importance of the two precursors, glucose to acetate, for acetyl CoA synthesis. Therefore, it seems likely that control of leptin secretion by ruminant adipocytes depends, to some extent, on acetate, and this should be addressed again in future studies.

We conclude that malonyl CoA may play a major role in the control of leptin production in bovine adipocytes, linking the flux of energy substrates to adipose homeostasis.

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