Effects of dietary lipoic acid on plasma lipid, in vivo insulin sensitivity, metabolic response to corticosterone and in vitro lipolysis in broiler chickens

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The present study consisted of two experiments. The first experiment was conducted to determine the effects of lipoic acid (ALA; 200 mg/kg) on plasma lipids and insulin sensitivity of whole-body tissue in broilers treated with or without corticosterone (5 mg/kg). Chickens received these agents from 2 to 5 weeks of age in a 2 x 2 factorial arrangement. Thereafter, from 39 to 42 d of age, insulin sensitivity was estimated using the euglycaemic and hyperinsulinaemic clamp technique. Experiment 2 examined whether ALA supplementation for 5 weeks (400 mg/kg) would alter short-chain acyl-CoA concentration in the liver and in vitro lipolysis of an adipose tissue slice, in relation to noradrenaline (10μM) supplementation. In experiment 1, ALA had no effect on the corticosterone-induced negative growth performance. ALA lowered plasma glucose level (P<0.05) and, in contrast, increased triacylglycerol level (P<0.05). These responses to ALA had, however, no interrelationship with corticosterone. The rate of glucose uptake of whole-body tissue was enhanced in the ALA-fed chickens (P<0.05), regardless of corticosterone treatment. In experiment 2, ALA increased only the plasma free glycerol concentration (P<0.01). The rate of free glycerol release from an adipose tissue slice was enhanced by ALA feeding (P<0.05) but was not affected by noradrenaline supplementation. This study suggests that ALA stimulates the insulin sensitivity of tissues regardless of corticosterone-dependent metabolism and that the ALA-induced fatty acid metabolism of broilers differs between the liver and adipose tissue.

Insulin sensitivity: Lipoic acid: Lipolysis

An active vitamin-like substance, α-lipoic acid (ALA), acts as a cofactor in enzyme complexes related to the oxidative decarboxylation of α-keto acids (Christensen, 1983). Recent studies have established that the administration of ALA enhances insulin-stimulated glucose uptake into and glucose oxidation in skeletal muscle in insulin-resistant obese rats (Henriksen et al. 1997; Streeper et al. 1997; Bustamante et al. 1998). The metabolic action of ALA affects not only glucose metabolism, but also lipid metabolism. The administration of ALA to normal rats has been reported to decrease serum triacylglycerol levels (Segermann et al. 1991). In rabbits with experimental atherosclerosis, reductions in serum total cholesterol and β-lipoproteins have been noted (Ivanov, 1974).

In domestic animals, however, there are few observations on the effects on glucose and lipid metabolisms. In broiler chickens, the author has found that the metabolic response of lipids to dietary ALA administration depends on the age-related metabolic state (Hamano et al. 1999) and that simultaneous increments in both plasma NEFA and triacylglycerol, an interesting response, occurred when the level of administration rose (Hamano, 2002). Khamaisi et al. (1999) also observed an increased plasma NEFA level from ALA administration in rats and suggested that this resulted from reduced hepatic fatty acid oxidation. As it is known that most fat synthesis in chickens occurs in the liver tissue (O’Hea & Leveille, 1969), the ALA-induced plasma lipid response may be associated with hepatic fatty acid utilisation, including the re-esterification of fatty acids (Hamano, 2002). The involvement of ALA in adipose tissue lipolysis has, however, not yet been investigated. Furthermore, in chickens, the influence of ALA administration on hormonal actions remains unclear. Hence, the present author initially found that ALA facilitated hyperglycaemic and lipolytic responses to an intravenous infusion of isoproterenol, a β-adrenergic agonist (Hamano et al. 1999, 2000).

In chickens, the responsiveness of the peripheral tissues to insulin is generally known to weaken with age (Vasilatos-Younken, 1986; Chou & Scanes, 1988), but it is unknown whether such insulin sensitivity of chickens increases with ALA feeding and leads to increased glucose uptake by the tissues. Moreover, corticosterone, which is a major glucocorticoid in chickens, impairs insulin signalling in the liver and skeletal muscle, resulting in insulin resistance (Dupont et al. 1999). The administration of corticosterone, in addition, increases adipose tissue accretion (Takahashi et al. 1993) with hyperlipidaemia (Malheiros et al. 2003) and stimulates hepatic glyconeogenesis (Webster, 2003). Thus, this hormone, which

Abbreviations: ALA, α-lipoic acid; EGC, euglycaemic and hyperinsulinaemic clamp; GIR, glucose infusion rate.

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affects insulin action and energy source distribution, may be associated with the ALA-induced metabolic response of chickens. On the other hand, an interrelationship between β-adrenergic agonists and ALA in fatty acid mobilisation has been found (Hamano et al. 2000; Hamano, 2002), but whether ALA aids the α-adrenergic response to noradrenaline or directly stimulates adipose tissue lipolysis is obscure.

The present study was therefore conducted to examine the effects of ALA on the plasma lipid response, glucose uptake and adipose tissue lipolysis in relation to several hormone actions of insulin, corticosterone and noradrenaline in broiler chickens.

Materials and methods

Animals and diets

Female broiler chicks (1 d old) (Ross strain; Shosan Shoji Co., Tokyo, Japan) were housed in electrically heated battery brooders. At 14 d old, chicks were transferred to wire cages and used for two experiments as described later. A commercial starter diet containing 230 g crude protein and 12.5 MJ metabolisable energy per kg was provided until 3 weeks of age, and thereafter a grower diet containing 180 g crude protein and 13.2 MJ metabolisable energy per kg was given. These diets and drinking water were provided ad libitum. Room temperature under continuous lighting was controlled at 25°C throughout the experiments. The present study was carried out in accordance with Guidelines for Animal Care and Experimentation of the Akita Prefectural College of Agriculture.

Experiment 1: Effects of dietary α-lipoic acid and corticosterone on growth performance, plasma metabolites and insulin sensitivity

Design. At 2 weeks old, chicks were randomly assigned to sixteen cages (60 × 75 × 75 cm) of four birds each. The cages were divided into four treatment groups for a 2 × 2 factorial arrangement (sixteen birds per treatment). ALA (race-mic type; Sigma Aldrich, St Louis, MO, USA) at 200 mg/kg and corticosterone (Sigma) at 5 mg/kg were added to the starter and grower diets. The first group was fed only the basal diet. The second and third groups were fed a diet containing ALA or corticosterone, respectively. The last was fed a diet with both agents. All chickens in each cage (n 4) were weighed at 36 d of age. Birds were then randomly selected from the cages (n 6), and blood (1 ml) was taken from a wing vein using a heparinised syringe. Plasma samples were obtained by centrifugation (2000 g) and assayed for glucose, triacylglycerol and NEFA. Thereafter, the chickens were deeply anaesthetised using pentobarbital sodium (Nembutal injection; Abbott Laboratories, North Chicago, IL, USA) to kill them; then, the left side of the breast muscle, liver and abdominal fat pad were removed and weighed (n 6). In addition, chickens from 39 to 42 d of age were used for the euglycaemic and hyperinsulinaemic clamp (EGC) experiment.

Euglycaemic and hyperinsulinaemic clamp experiment. The EGC technique has been used to estimate in vivo insulin sensitivity during physiological changes. The technique possesses the advantage that the tissue uptake of glucose stimulated by exogenous insulin is determined at a steady blood glucose concentration (Chou & Scanes, 1988).

In preparation for the EGC experiment, randomly selected chickens (n 5) were covered with a rubber suit made in the laboratory. Two medical cannulas (inner diameter 0.48 mm, outer diameter 0.68 mm; Nipro, Osaka, Japan) were inserted into the chicken’s leg veins under local anaesthesia (5 g/l lido-caine hydrochloride; Astra Japan Ltd, Osaka, Japan). The birds were then transferred to a two-shelf metal wire rack. The rubber suit containing the chickens was connected to rubber bands hanging from the upper shelf of the rack to hold the chickens in a standing position on the bottom shelf during the experiment. One cannula was used for blood sampling, and the other was connected to a silicon tube with both a syringe pump (Nipro) for insulin infusion and a peristaltic pump (Atto Co., Tokyo, Japan) for glucose infusion. The interior of the blood-sampling cannula was filled with saline containing heparin.

Chou & Scanes (1988) found physiological changes in insulin sensitivity in chickens using the EGC technique with an infusion rate of bovine insulin of 22 mU/kg per min. In the present study, bovine insulin (Sigma) dissolved in saline (pH 2.8) was continuously infused from a syringe pump at the rate of 20 mU/kg per min for 2 h. In a preliminary experiment, a continuous infusion of bovine insulin at 20 mU/kg per min for 2 h achieved a 48% reduction in plasma glucose concentration in 6-week-old male chickens (means of 60–120 min; n 3) compared with the saline-infused birds (n 3).

During the EGC experiment, blood (1 ml) was drawn at 10-min intervals using a heparinised syringe. The sampling was initiated 20 min before infusion of the bovine insulin. The blood samples were immediately used for blood glucose assay and thereafter centrifuged to obtain plasma. Plasma samples were stored at −20°C until glucose assay to express the data as plasma concentrations. To prevent blood glucose reduction following the infusion of exogenous bovine insulin and to maintain the basal concentration of blood glucose (euglycaemia), D-glucose solution (200 g/l) dissolved in saline was exogenously and concomitantly infused using a peristaltic pump at variable rates. The infusion rate was adjusted immediately after blood glucose was analysed (within 2 min) to maintain the euglycaemic state, which was in fact achieved during approximately the first 60 min.

To calculate the glucose infusion rate (GIR; mg/kg per min), the weight of the infused glucose solution was recorded at 10-min intervals, using an electronic scale. The GIR was calculated every 10 min and converted from the weight of the solution infused and its specific gravity (1.083 of the measured value). Because the chicken was in a stable state of euglycaemia and hyperinsulinaemia under glucose infusion during the latter 60 min period, the GIR from 60 to 120 min was used for statistical estimation of the insulin sensitivity. Accordingly, the GIR, which was required to maintain the basal glucose concentration, was considered to be the rate of glucose uptake in the response of all body tissues to exogenous bovine insulin.

Analyses. Blood and plasma concentrations of glucose were determined by an automatic analyser (M110; Sakura Inc., Tokyo, Japan) with an enzymatic membrane sensor using pyranose oxidase. This analyser was able to determine...
the concentration of a blood glucose sample within 60 s. Other plasma metabolites were determined with colorimetric and enzymatic assay kits for triacylglycerol (Sanko Chemical, Tokyo, Japan) and NEFA (Kyowa Medex, Tokyo, Japan). Data were statistically analysed with two-way ANOVA (StatView; Abacus Concepts Inc., Berkeley, CA, USA).

Experiment 2: Involvement of α-lipoic acid in concentration of short-chain acyl-CoA in liver and in vitro lipolytic response of adipose tissue

Design. Broiler chicks (14 d old) were transferred to eight wire cages containing four birds each; half the cages were assigned to the ALA treatment group. Chicks were fed on the diet supplemented or not supplemented with ALA at 400 mg/kg. At 50 d of age, chickens were randomly selected, and blood (1 ml) was taken from a wing vein using a heparinised syringe (1 ml; n = 6). Plasma samples obtained from blood centrifugation were stored at −20°C until subsequent analysis for plasma glucose, NEFA, triacylglycerol and free glycerol. The chickens were then killed using pentobarbital sodium (Nembutal). The liver was removed and frozen with liquid nitrogen. Thereafter, the tissue samples were immediately homogenized with ice-cold sulfosalicylic acid (50 g/l) containing 0.5 mm thickness were prepared using a vibrating blade microtome (VT 1000S; Leica Instruments GmbH, Nussloch, Germany). A 75–100 mg slice was transferred to 3 ml incubation buffer (pH 7-4) containing 20 mM-d-glucose and 20 mM-HEPES and embedded in 30 g/l agarose (type VII-A, low gelling temperature; Sigma) dissolved in the buffer (less than 35°C) and cooled in ice. Tissue slices of 0.5 mm thickness were prepared using a vibrating blade microtome. The tube was sealed with a screw cap under an atmosphere of 5% CO2 in O2 and was incubated in duplicate for 2 h in a shaking water bath maintained at 37°C. The incubation was terminated by placing the tube in an ice bath. The buffer solution incubated with the adipose tissue slice was collected and stored at −20°C until free glycerol assay.

Analyses. Plasma glucose, NEFA and triacylglycerol were determined with enzymatic assay kits as described earlier. To estimate the lipolytic response, free glycerol in the plasma and buffer incubated with the adipose tissue slice was assayed with a fluorometric method using glycerol dehydrogenase (Boobis & Maughan, 1983). Tissue concentrations of short-chain acyl-CoA were determined using an HPLC method (Demoz et al. 1995). Data from plasma metabolite and short-chain acyl-CoA concentrations were statistically analysed using a Student’s t test (StatView). The release rate of in vitro free glycerol from an adipose tissue slice was determined by ANOVA as a 2 × 2 factorial arrangement.

Results

Experiment 1: Effects of dietary α-lipoic acid and corticosterone on growth performance, plasma metabolites and insulin sensitivity

The effects of dietary ALA and corticosterone administrations on growth performance are shown in Table 1. Dietary treatment with corticosterone was apparently detrimental to growth performance. Body weight gain was significantly decreased (P < 0.001) with hormone administration by 13% but was not affected by ALA supplementation. The breast muscle weight in the corticosterone-treated group was also lower (P < 0.05) than that in the untreated group. Supplementation with ALA had no effect on breast muscle weight. In spite of the retardation in body weight gain with the corticosterone treatment, corticosterone increased abdominal fat weight by approximately 1.8 times (P < 0.001) compared with controls. However, dietary ALA did not affect abdominal fat weight regardless of corticosterone treatment.

Plasma concentrations of glucose, triacylglycerol and NEFA are shown in Table 2. Whereas corticosterone treatment did not affect the plasma glucose level, a 17% reduction in

<table>
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<th>Corticosterone (mg/kg)</th>
<th>ALA (mg/kg)</th>
<th>Mean</th>
<th>SE</th>
<th>n†</th>
<th>Mean</th>
<th>SE</th>
<th>n†</th>
<th>Mean</th>
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<table>
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<tr>
<th>Statistical significance (ANOVA)</th>
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<tbody>
<tr>
<td>Corticosterone</td>
</tr>
<tr>
<td>ALA</td>
</tr>
<tr>
<td>Corticosterone × ALA</td>
</tr>
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</table>

† Values represent means for four replicates of four birds per cage.

For details of diets and procedures, see. p. 1095.

Table 1. Effects of α-lipoic acid (ALA) and corticosterone on final body weight, body weight gain, tissue weights of breast muscle and abdominal fat in broiler chickens

(Mean values with their standard errors)
plasma glucose was observed in the ALA-fed chickens (P<0.05). In terms of plasma lipids, dietary corticosterone increased both plasma triacylglycerol (P<0.001) and NEFA (P<0.001) by approximately 2.2 and 1.4 times, respectively. Concomitantly, the administration of ALA enhanced plasma triacylglycerol level by 39% (P<0.05) but did not enhance plasma NEFA (P > 0.05).

Plasma glucose concentrations and GIR during the EGC experiment are shown in Fig. 1. During infusion of the bovine insulin, exogenous glucose solution was infused at a variable rate that approximately maintained the basal plasma glucose concentration. The GIR, which was estimated to be the rate of glucose uptake every 10 min, was gradually increased with the continuous infusion of bovine insulin and reached a steady state from approximately 60–120 min. The GIR values in the control and corticosterone-fed groups were 41.0 (SE 2.4; n 10) and 36.8 (SE 2.0; n 10) mg/kg per min, respectively (P>0.05). On the other hand, the GIR in the birds supplemented with ALA was higher than that of control from the first 60 min period regardless of corticosterone treatment (Fig. 1). The GIR in the latter 60-min period was significantly (P<0.001) higher in the ALA-fed chickens (44.4 (SE 1.4) mg/kg per min; n 10) than in controls (33.3 (SE 1.7) mg/kg per min; n 10), as shown by ANOVA. Thus, these results indicate that ALA stimulated the glucose uptake of whole-body tissues in response to exogenous insulin in the chickens but that this response was not associated with corticosterone.

**Experiment 2: Involvement of α-lipoic acid in concentration of short-chain acyl-CoA of liver and in vitro lipolytic response of adipose tissue**

Plasma metabolite concentrations are shown in Table 3. The plasma glucose concentration in the ALA-fed chickens was not significantly different from that of controls. Plasma triacylglycerol concentrations, except for methylmalonyl-CoA, were unchanged with ALA feeding. In addition, neither free CoA nor the molar ratio of free CoA:acetyl-CoA was affected by ALA feeding. On the other hand, the methylmalonyl-CoA concentration was 29% higher in the ALA-fed chickens than in controls (control 15.9 (SE 0.8, n 6) v. ALA 20.5 (SE 1.2, n 6) nmol/g fresh tissue; P<0.01).

The rate of free glycerol release from adipose tissue slices is shown in Fig. 2. The presence of 10 μM-noradrenaline in the incubation buffer had no significant effect on the free glycerol release (control 3.57 (SE 0.57, n 10) v. noradrenaline 4.92 (SE 1.01, n 8) μmol/g per 2 h). Although no significant interrelationship between ALA and noradrenaline was observed, the rate of free glycerol release in the ALA-fed group rose significantly compared with that in controls (control 3.00 (SE 0.50, n 9) v. ALA 5.35 (SE 0.85, n 9) μmol/g per 2 h; P<0.05). These results showed that ALA had a lipolytic action in adipose tissue but was not associated with noradrenaline supplementation.

**Discussion**

**Growth performance due to α-lipoic acid and corticosterone**

The present results showing that ALA administration alone had no effect on body weight gain or tissue weight (experiment 1) are consistent with previous reports (Hamano et al. 1999; Hamano, 2002). The present study did not measure feed intake and feed efficiency, but the energy intake of ALA-fed chickens would be unlikely to affect the plasma metabolites because no change in feed intake and feed efficiency of ALA-fed chickens was observed (Y Hamano, unpublished results). Thus, this study, at least, indicated that ALA had no preventable effect on corticosterone-induced negative growth performance.

**Plasma glucose and insulin sensitivity of whole body**

The hypoglycaemic effects of ALA associated with enhanced insulin action have been well demonstrated in mammals (Henriksen et al. 1997; Streepers et al. 1997; Bustamante et al. 1998). In the present study, a significant reduction in plasma glucose level in the ALA-fed chickens was also shown in experiment 1, but not observed in

**Table 2. Effects of α-lipoic acid (ALA) and corticosterone on plasma concentrations of glucose, NEFA and triacylglycerol in broiler chickens**

(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Corticosterone (mg/kg)</th>
<th>ALA (mg/kg)</th>
<th>Glucose (mmol/l)</th>
<th>NEFA (μmol/l)</th>
<th>Triacylglycerol (μmol/l)</th>
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<td>n</td>
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<td>200</td>
<td>12.8</td>
<td>0.4</td>
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Statistical significance (ANOVA)

Corticosterone NS *** NS
ALA * NS NS
Corticosterone × ALA NS NS NS

Mean values were significantly different: *P < 0.05; ***P < 0.001.

For details of diets and procedures, see p. 1095.
experiment 2. The plasma glucose decrease caused by ALA would be, in part, attributable to enhanced insulin sensitivity of the tissues. In rats, ALA has been reported to inhibit hepatic gluconeogenesis (Blumenthal, 1984). Whether the hypoglycaemic effect of ALA was dependent on hepatic glycogenolysis or glucose production in the liver and kidney remains unclear.

The present study showed that ALA enhanced the insulin sensitivity of whole-body tissues. In the EGC experiment, in which arterial blood glucose is monitored to maintain euglycaemia during glucose and insulin infusion, the GIR is regarded as being equal to the rate of tissue uptake of glucose (DeFronzo et al. 1979; Chou & Scanes, 1988). In this study, blood was taken from a leg vein because of technical

![Fig. 1. Effects of dietary lipoic acid (ALA) on plasma glucose and glucose infusion rate (GIR) during the euglycaemic clamp in chickens treated without corticosterone (A) or chickens treated with corticosterone (B). Values are means for five birds with standard errors represented by vertical bars.  O,  group fed the basal diet;  ALA-fed group. BW, body weight. Significance levels from ANOVA as a 2 × 2 factorial arrangement: corticosterone,  P > 0.05; ALA,  P < 0.001; interaction,  P > 0.05. For details of diets and procedures, see p. 1095.](image-url)
problems. To correctly estimate the glucose uptake from the GIR, blood taken from an artery is preferable in consideration of the arteriovenous difference in blood glucose concentration. Hence, it is undeniable that the GIR obtained from this study was, overall, overestimated. In addition, the extent of its overestimation would be different between controls and the ALA-treated group, even though the EGC technique was performed under the same experimental conditions for both treatment groups. Therefore, the GIR determined to assess insulin sensitivity should be considered to be an appearance rate in this study.

In terms of regulation of insulin action, corticosterone has been reported to reduce signalling to the liver and skeletal muscle, resulting in insulin resistance (Dupont et al. 1999). The dietary corticosterone in this study did not affect the insulin sensitivity of chickens. Thus, although the interrelationship of ALA with the corticosterone-related insulin action was not clear, the present study suggests that stimulation of insulin sensitivity with ALA occurs not only in mammals, but also in chickens.

Responses of plasma lipids to α-lipoic acid

The finding that ALA administration increased plasma triacylglycerol concentration (experiment 1) is consistent with a previous report, which, in addition, found a concomitantly enhanced plasma NEFA response (Hamano, 2002). The administration of ALA to normal rats has been reported to decrease serum triacylglycerol (Segermann et al. 1991). In rabbits with experimental atherosclerosis, reductions in serum total cholesterol and β-lipoproteins have been found (Ivanov, 1974). Dietary ALA at a level of 50 mg/kg also lowers plasma triacylglycerol in broilers (Hamano et al. 1999). It is well known that most fat (fatty acid) synthesis in chickens occurs in the liver (O’Hea & Leveille, 1969). Thus, blood triacylglycerol responses to ALA, especially in chickens, would be closely related to the fatty acid metabolism of the liver and adipose tissue. In this study, ALA had no effect on plasma NEFA, even when increased by corticosterone that would result from the stimulated fatty acid mobilisation.
On the other hand, as shown in experiment 2, the plasma free glycerol concentration and in vitro release rate of free glycerol from adipose tissue (discussed later), which would be a more likely index of lipolysis, increased in the ALA-fed chickens. Khamaisi et al. (1999) noted that short-term ALA treatment increased plasma NEFA in fasted rats, but they suggested that ALA inhibited hepatic fatty acid oxidation. Even if ALA accelerated fatty acid oxidation in the liver, the distribution of acetyl-CoA derived from β-oxidation in the Krebs cycle might be limited. Hence, the plasma triacylglycerol increase caused by ALA feeding might be attributed to an indirect stimulation of hepatic fatty acid (malonyl-CoA) biosynthesis, although no change in the concentrations of acetyl-CoA and malonyl-CoA was shown in the liver (experiment 2). In addition, Scaife et al. (1978) found that the presence of methylmalonyl-CoA in chicken liver in vitro inhibited fatty acid synthesis. Neither plasma triacylglycerol nor other short-chain acyl-CoA intermediates were altered in the ALA-fed chickens (experiment 2). Thus, the hepatic methylmalonyl-CoA accumulation in this study might instead be associated with branched-chain amino acid oxidation.

**Lipolytic response to α-lipoic acid**

In rat hepatocytes, ALA has been shown to inhibit glyconeogenesis-derived from free glycerol (Blumenthal, 1984). In relation to the increased plasma glycerol, the present study also confirmed ALA-induced lipolysis by determining the release rate of free glycerol from adipose tissue slices (experiment 2). Consequently, a stimulatory effect of ALA on in vitro adipose tissue lipolysis was observed. Previous studies indicated that ALA increased the lipolytic response to the intravenous injection of two β-adrenergic agonists, isoproterenol and clenbuterol, in broiler chickens (Hamano et al. 2000; Hamano, 2002). ALA did not, however, aid or elicit noradrenaline-related lipolysis, which is, in general, insensitive in chicken adipose tissue (Wellenreiter, 1991). The ALA-stimulated lipolysis was therefore an independent action under the in vitro condition, but the mode of the action in the response of lipolysis remains uncertain. Taken together with the increased plasma glycerol, ALA supplementation may be a possible method to control adipose tissue accretion. However, consideration of hepatic fatty acid metabolism in response to ALA will be necessary to control adipose tissue accretion.

**Conclusions**

Dietary ALA supplementation was found to increase the insulin-related glucose uptake of peripheral tissues in chickens. Moreover, in chickens, ALA facilitated in vitro lipolysis in adipose tissue and, in contrast, increased plasma triacylglycerol level, but had no interrelationship with corticosterone or noradrenaline. Therefore, the present study suggests that ALA enhances insulin sensitivity in chickens as in mammals, and that the effect on fatty acid metabolism differs between liver and adipose tissue.

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**References**


