The Role of Glucagon in Regulating Chicken Hepatic Malic Enzyme and Histidase Messenger Ribonucleic Acid Expression In Response to an Increase in Dietary Protein Intake

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ABSTRACT Increased dietary protein intake rapidly (3 h) decreases hepatic malic enzyme and increases hepatic histidase mRNA expression in broiler chicks. A series of experiments was conducted to determine the role that glucagon or a specific mixture of dietary amino acids might have in regulating the rapid changes in mRNA expression of these enzymes, when dietary protein intake is increased. Three hours after the injection of glucagon (240 μg/kg of BW) into the brachial vein of broiler chicks, hepatic malic enzyme mRNA expression was significantly lower and hepatic histidase mRNA expression was significantly greater than the level detected in saline-injected chicks. In addition, broiler chicks fed a high (40 g/100 g of diet) protein diet had significantly higher plasma glucagon levels at 1 and 3 h after initial access to this diet than broiler chicks fed a basal (22 g/100 g of diet) protein diet. The plasma glucagon concentration, however, was not different between the chicks fed the 2 dietary protein levels at 2 h after the initial access to the 2 diets. When a mixture of indispensable or dispensable amino acids was added to the basal diet to equal the concentrations of the individual indispensable or dispensable amino acids in the high protein diet, hepatic mRNA expression of malic enzyme and histidase were intermediate to the expression found in chicks fed the basal and high protein diet. The results indicate that glucagon may mediate the changes in the mRNA expression of malic enzyme and histidase in response to dietary protein intake and that total amino acid intake rather than the ingestion of specific amino acids regulates the mRNA expression of malic enzyme and histidase in chicks.

Key words: broiler chick, amino acid, temporal change

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INTRODUCTION

The activity of hepatic malic enzyme is positively correlated with the rate of fatty acid synthesis, the percentage of body fat, and the percentage of abdominal fat in chicks (Yeh and Leveille, 1969; Pfaff, 1977; Tanaka et al., 1983; Grisoni et al., 1991). Adams and Davis (2001) reported that switching chicks from a basal protein diet (22 g/100 g of diet) to a low (13 g/100 g of diet) or high (40 g/100 g of diet) protein diet resulted in a rapid (3 h) change in the expression of the mRNA for malic enzyme. A switch to a low protein diet increased the level of malic enzyme mRNA, whereas feeding a high protein diet decreased its level. The changes in malic enzyme mRNA expression were associated with subsequent changes in malic enzyme activity and liver total lipid concentration.

In contrast, the activity of hepatic histidase increases when chicks are fed increasing dietary concentrations of protein (Scott and Austic, 1982; Keene and Austic, 2001). In chicks, hepatic histidase mRNA expression increases as early as 3 h after the ingestion of a high protein diet (Chendrimada and Davis, 2005). Interestingly, when chicks are fed a nutritionally adequate diet supplemented with L-histidine, hepatic histidase mRNA expression was not altered (Chendrimada and Davis, 2005).

The mechanism by which dietary protein intake rapidly modifies hepatic malic enzyme and histidase mRNA expression is not understood, but glucagon may play a critical role. In mammalian species, an intravenous infusion of amino acids or an increase in the concentration of dietary protein elevated plasma glucagon levels (Ohneda et al., 1968; Kuhara et al., 1991; Tovar et al., 2002). In addition, in red-tailed hawks, there was a significant increase in the plasma concentration of glucagon 1 h after an oral infusion of a mixture of indispensable and dispensable amino acids (Minick et al., 1996). Furthermore, Lefevre et al. (1999) reported that the addition of glucagon to cultured chick hepatocytes significantly depressed malic enzyme mRNA levels. Finally, rat hepatic histidase activity is regulated by dietary protein at the pretranslational level, and glucagon is involved in the induction of histidase mRNA expression (Torres et al., 1998; Tovar et al., 2002).

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Therefore, the present research was conducted to determine if glucagon plays a role in the rapid alteration of hepatic mRNA concentrations for malic enzyme and histidase when chicks are fed different levels of dietary protein. In addition, the role of dietary indispensable and dispensable amino acids in regulating malic enzyme and histidase mRNA expression was also investigated.

MATERIALS AND METHODS

General Animal Care

Day-old mixed-sex broiler chicks (Ross × Ross, ConAgra, Athens, GA) were raised in thermostatically controlled, electrically heated battery brooder cages with wire floors. The cages were lighted for 24 h/d, and the chicks had free access to a practical chick starter diet and water. Seven days after hatching, the chicks were sorted by weight, and those with extreme weights were discarded. The remaining chicks were randomly assigned to experimental groups to achieve a similar weight distribution among all pens. The chicks were then fed a glucose monohydrate and isolated soybean protein-based basal diet containing 22 g of protein/100 g of diet for an acclimation period of 4 or 5 d, after which they were given access to the experimental diets. The semipurified diet used in the present experiments was the adjusted basal diet reported previously (Adams and Davis, 2001). For convenience, the adjusted basal diet will be designated as the basal diet in this manuscript. At the end of each experiment, feed consumption and body weight gain were determined for each pen. The chicks were killed by cervical dislocation to obtain liver samples for RNA extraction. The Institutional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

Experiments 1 and 2

The goal of these 2 experiments was to determine if an exogenous supplement of glucagon could rapidly alter hepatic malic enzyme and histidase mRNA expression in broiler chicks. After sorting, the chicks were randomly assigned to 24 pens each consisting of 2 birds. Following the acclimation period, the pens were split into 2 groups. The chicks in 1 group were injected (brachial vein) with 240 μg of glucagon/kg of BW, and the birds in the other group were injected with an equivalent volume of saline. The glucagon used in these experiments was obtained from porcine pancreas cells (Sigma, St. Louis, MO). After the injection, the birds continued to have free access to the basal diet and water for either 1.5 or 3 h. Therefore, there were 6 replicate pens of 2 birds each for each experimental treatment. At the end of each experimental period, a sample of approximately 250 mg was taken from the left lobe of the liver of each chick and combined by pen for RNA extraction.

The protocol for Experiment 2 was similar to that of Experiment 1, except that the effect of glucagon on the mRNA expression of malic enzyme and histidase was examined only at 3 h and there were only 4 replicate pens of 2 birds each per treatment.

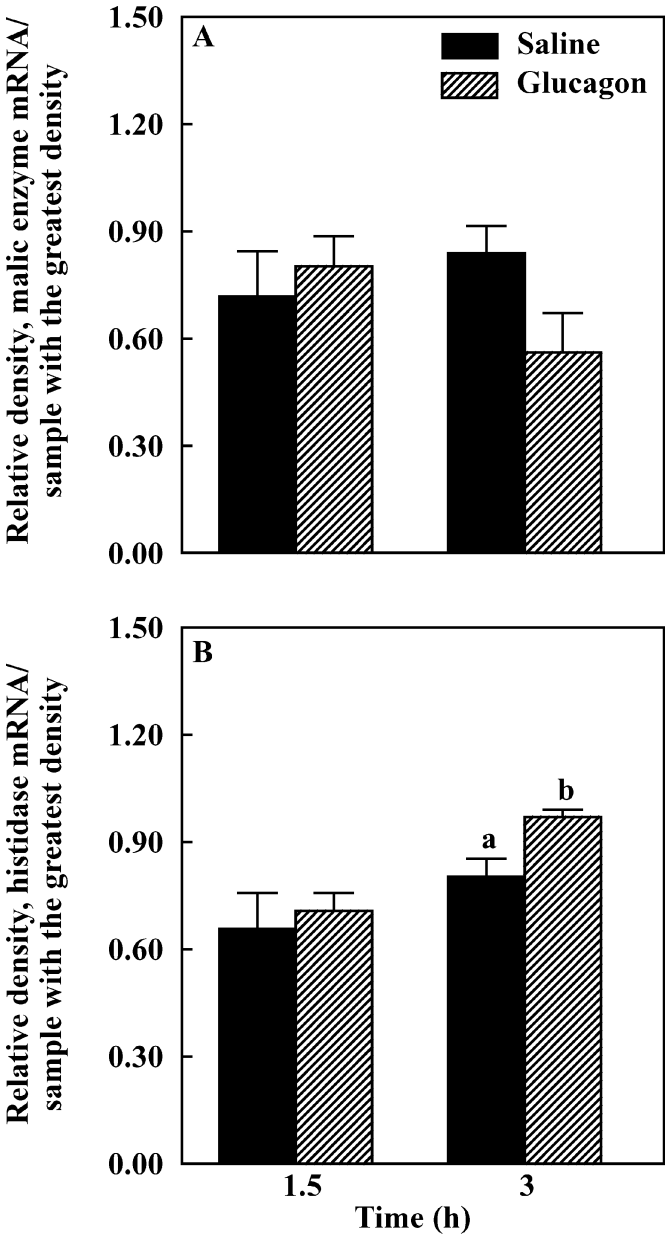

Figure 1. The relative density of hepatic malic enzyme (A) and hepatic histidase mRNA (B) of chicks at 1.5 or 3 h after brachial vein injection of either glucagon or saline. Values are means ± SEM, n = 6 replicate pens for glucagon- or saline-injected birds. [a,b]Means at a given time with different letters differ, $P < 0.05$. Note that the relative densities of malic enzyme and histidase mRNA to one another are specific for each time point and that all statistical comparisons are within a given time period.

Experiment 3

This experiment was conducted to determine if an increase in dietary protein consumption rapidly changed the plasma glucagon concentration in chicks. At the end of the acclimation period, the chicks were deprived of food for 2 h (0500 to 0700) and then given free access to

either the basal (22 g of protein/100 g of diet) or a high protein diet (40 g of protein/100 g of diet) for 0, 1, 2, or 3 h. The 2-h food deprivation was done to help insure initial consumption of the experimental diets. The ingredient composition of the high protein diet has been reported previously (Adams and Davis, 2001). For each dietary treatment, there were a total of 9 replicate pens of 2 birds each.

At the end of each experimental period, blood was collected from the brachial vein of each bird and immediately placed into individual glass vacutainers (Becton, Dickinson and Co., Franklin Lakes, NJ) containing EDTA as an anticoagulant. The vacutainers were then placed in ice. For the 3-h experimental period, liver samples were collected from the chicks of each pen as described for Experiment 1. The liver samples were collected at this time for subsequent RNA extraction to allow Northern blot analyses to confirm previously documented (Adams and Davis, 2001) differences in mRNA expression of malic enzyme in chicks fed the 2 dietary levels of protein.

Blood samples were centrifuged at 1,000 × *g* at 4°C for 10 min. Plasma was collected from each sample and then frozen at –80°C. Plasma glucagon concentrations were determined by RIA (DA Glucagon Kit, ICN Biochemicals, Irvine, CA). The glucagon RIA was performed following the manufacturer's instructions using duplicate samples from each pen. Each duplicate sample for a pen contained a 100-μL aliquot of plasma from each chick.

Experiments 4 and 5

These experiments were conducted to determine the role of the indispensable and dispensable amino acids in the regulation of the mRNA expression of malic enzyme and histidase. After sorting, the chicks were randomly assigned to 24 pens each consisting of 2 birds. Following the acclimation period, the pens were split into 4 equal groups. The chicks in one group were maintained on the basal diet, and the chicks in another group were fed the high (40 g/100 g of diet) protein diet. The chicks in the remaining 2 groups were fed either the basal diet supplemented with indispensable (**IAA**[2]) or dispensable amino acids (**DAA**[3]). When the IAA or DAA supplements were added the basal diet, the concentration of the individual indispensable or dispensable amino acids of the supplemented diet equaled the concentrations of these amino acids in the high protein diet. The chicks were given free access to the experimental diets for 6 h (Experiment 4) or 24 h (Experiment 5). At the end of each experiment, total feed consumption was determined for each pen, and liver samples were collected from each bird and combined by pen for RNA extraction.

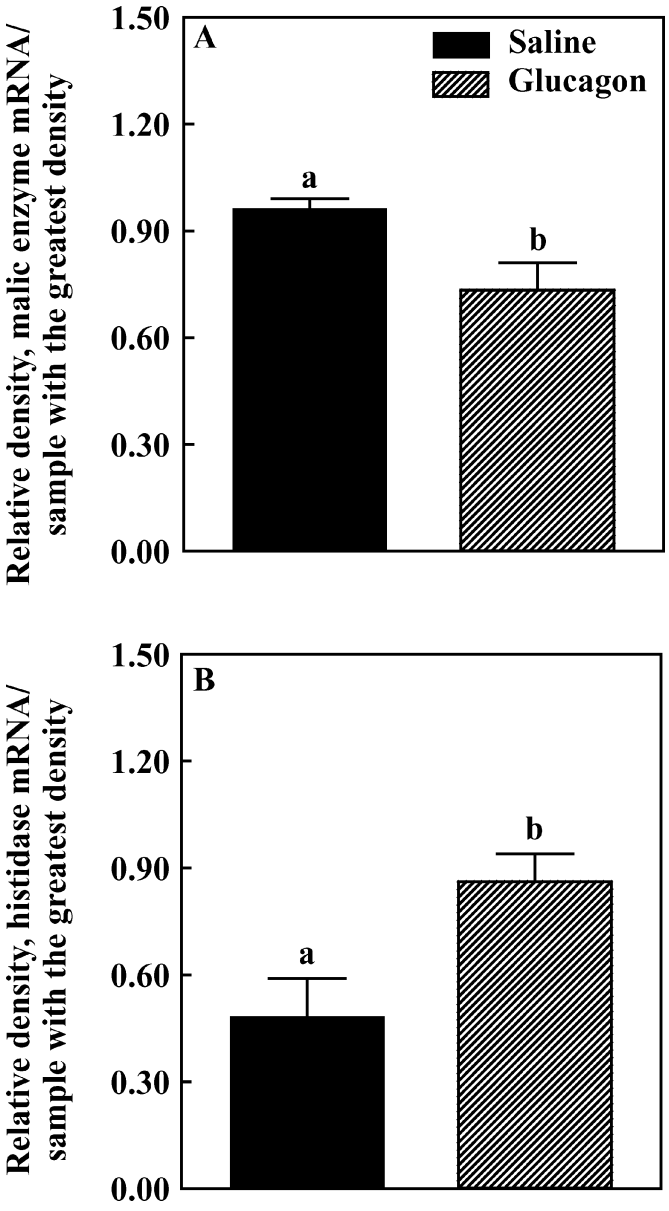

Figure 2. The relative density of hepatic malic enzyme (A) and hepatic histidase (B) mRNA of chicks 3 h after brachial vein injection of either glucagon or saline. Values are means ± SEM, n = 4 replicate pens. [a,b]Means with different letters differ, $P < 0.05$.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from liver samples pooled by pen using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA (40 μg/sample) was electrophoresed on an agarose/formaldehyde gel and then transferred to a nylon membrane as

[2]The IAA supplement provided the following (in g/100 kg of diet): arginine 1.258, glycine 1.025, histidine 0.432, isoleucine 0.808, leucine 1.352, lysine 1.033, methionine 0.715, phenylalanine 0.864, threonine 0.620, tryptophan 0.225, and valine 0.826. The addition of the IAA supplement was at the expense of sand in the original adjusted basal diet (5), and to keep the diet isocaloric to the basal and high protein diets, the corn oil of the original diet was reduced from 11.50 to 8.10 g/100 g of diet.

[3]The DAA supplement provided the following (in g/100 kg of diet): alanine 0.714, aspartic acid 1.916, cysteine 0.207, glutamic acid 3.155, proline 0.845, serine 0.864, and tyrosine 0.620. The addition of the IAA supplement was at the expense of sand in the original adjusted basal diet (5), and to keep the diet isocaloric to the basal and high protein diets, the corn oil of the original diet was reduced from 11.50 to 8.10 g/100 g of diet.

Table 1. Plasma glucagon concentration and food consumption of chicks fed the basal or high protein diets for 0, 1, 2, or 3 h (Experiment 3)

Time (h)	Treatment	Plasma glucagon (pg/mL)[1]	Food consumption (g/chick)[1]
0	—	1,599 ± 242	—
1	Basal	785 ± 98[a]	3.33 ± 0.19[a]
	High	1,289 ± 197[b]	2.10 ± 0.22[b]
2	Basal	1,503 ± 83	5.00 ± 0.33[a]
	High	1,482 ± 130	3.22 ± 0.22[b]
3	Basal	1,385 ± 153[a]	6.00 ± 0.66[a]
	High	2,061 ± 188[b]	4.00 ± 0.50[b]

[a,b]Means at a given time lacking a common superscript differ ($P < 0.05$). Note that all statistical comparisons are specific for the given experimental period.

[1]Values are means ± SEM, n = 9 replicate pens.

previously described (Davis and Johnson, 1998). Duck malic enzyme (Glynias et al., 1984), chicken histidase (Chendrimada and Davis, 2005), and chicken glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**; Davis and Johnson, 1998) cDNA clones were prepared and labeled with ^{32}P for Northern blot analysis as previously described (Davis and Johnson, 1998). The hybridization and densitometry procedures followed those described previously (Davis and Johnson, 1998). There were 2 blots for each experimental time period, and the replicate samples for each dietary treatment were divided equally between the 2 blots. The 2 blots were hybridized at the same time, and exposed together on the same film. The blots were stripped (Davis and Johnson, 1998) of the previously hybridized probe before being hybridized with a subsequent probe. The order of the hybridizations was malic enzyme, histidase, and GAPDH. The relative mRNA expression of malic enzyme or histidase was determined for the samples of each blot by calculating the signal intensity of each sample relative to the strongest signal, which was assigned a value of 1. Before calculation of relative malic enzyme or histidase mRNA levels, GAPDH mRNA expression was used to correct the malic enzyme and histidase values for equality of RNA loading and transfer for each blot.

Statistical Analyses

Data from each experiment were subjected to ANOVA according to the GLM procedure of SAS (Version 8.0, SAS Institute, Cary, NC). Fisher's pairwise comparison procedure was used in Experiments 1 to 3, and Tukey's multiple-comparison procedure was used in Experiments 4 and 5 to detect significant differences among the treatments (Neter et al., 1990). Differences were considered significant when P-values were <0.05.

RESULTS

Experiments 1 and 2

Malic enzyme and histidase mRNA expression were not significantly different between chicks injected with either glucagon or saline at 1.5 h (Figure 1). At 3 h, malic enzyme mRNA expression in the glucagon-injected birds tended (P-value = 0.077) to be lower than saline-injected birds (Figure 1). Hepatic histidase mRNA expression was significantly greater in the glucagon-injected birds than in the saline-injected controls at 3 h (Figure 1). Food consumption did not differ among the chicks injected with saline or glucagon at 1.5 or 3 h (data not shown).

In Experiment 2, chicks injected with glucagon had a significantly lower expression of malic enzyme mRNA and a significantly greater expression of histidase mRNA than chicks injected with saline (Figure 2). Food consumption did not differ between the control chicks and those injected with glucagon (data not shown).

Experiment 3

The birds fed the basal diet consumed more food than the birds fed the high protein diet for all of the experimental time periods (Table 1). The concentration of plasma glucagon (pg/ml) was significantly greater at 1 and 3 h, but not at 2 h in chicks fed the high protein diet when compared with chicks fed the basal protein diet (Table 1). The mean ± SEM relative mRNA values at 3 h for malic enzyme were 0.73 ± 0.06 and 0.35 ± 0.04 and for histidase were 0.23 ± 0.09 and 0.71 ± 0.08 for the basal and high protein diets, respectively. Expression of malic enzyme mRNA was significantly lower, and the expression of histidase mRNA was significantly higher in chicks fed the high protein diet when compared with chicks fed the basal diet at 3 h.

Experiment 4

Food consumption (mean ± SEM, grams of feed consumed per chick) for chicks fed the basal, high, IAA-supplemented, and DAA-supplemented diets was 14.0 ± 1.5, 10.7 ± 0.3, 11.5 ± 0.7, and 10.4 ± 0.4, respectively. The only significant ($P < 0.05$) differences in food consumption were between the chicks fed the basal diet and those fed the high and DAA-supplemented diets.

Because of limitations in the number of RNA samples that could be electrophoresed in one gel, RNA samples from the chicks fed the IAA- and the DAA-supplemented diets were each electrophoresed with the RNA samples

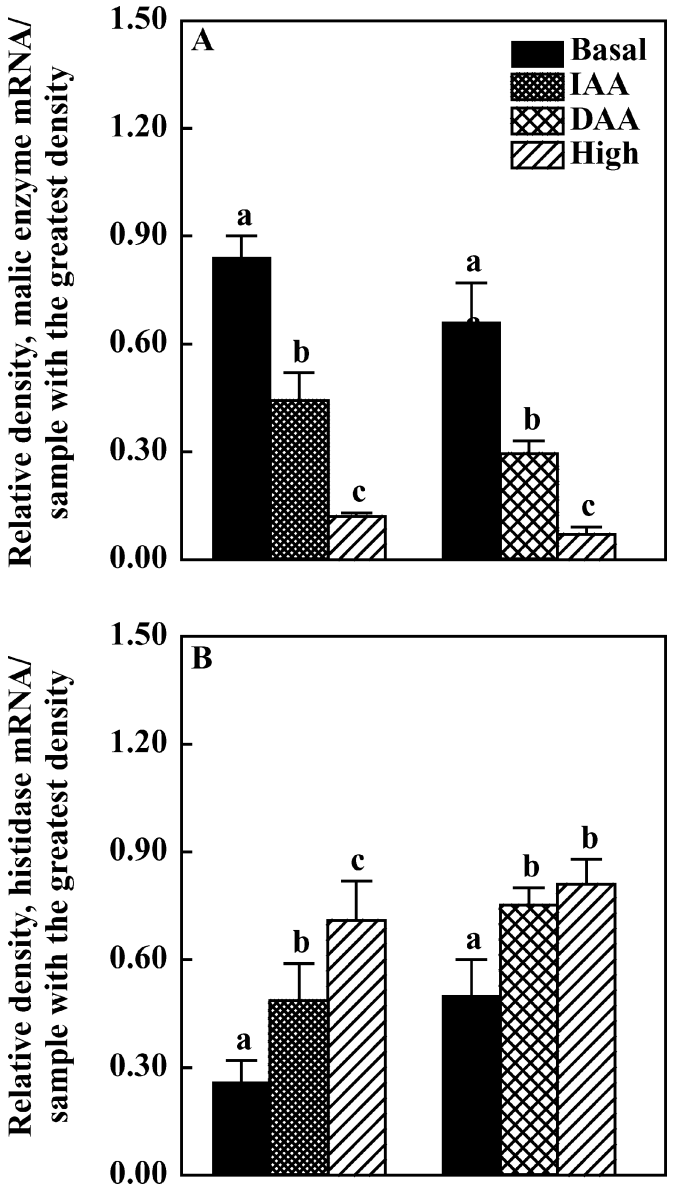

Figure 3. The relative density of hepatic malic enzyme (A) and histidase (B) mRNA of chicks fed for 6 h the basal diet, high protein diet, or the basal diet supplemented with indispensable (IAA) or dispensable (DAA) amino acids. Values are means ± SEM, n = 6 replicate pens. [a–c]Means with different letters differ, $P < 0.05$. Note that the relative densities of malic enzyme and histidase mRNA to one another are specific for each dietary group and that all statistical comparisons are within a given dietary group.

obtained form the chicks fed the basal and high protein diet on separate gels. At 6 h, malic enzyme mRNA expression in chicks fed the IAA- and the DAA-supplemented diets was significantly lower than in chicks fed the basal diet but not as low as in chicks fed the high protein diet (Figure 3A). Chicks fed the high protein diet, the IAA-supplemented diet, or the DAA-supplemented diet had significantly greater hepatic histidase mRNA expression than chicks fed the basal diet (Figure 3B).

Experiment 5

Food consumption at 24 h in chicks fed the basal diet, the high protein diet, or the basal diet supplemented with IAA or DAA was (mean ± SEM) 49.5 ± 3.5, 40.3 ± 3.9, 43.7 ± 2.2 and 53.0 ± 1.2 g/chick, respectively. The only significant difference in food consumption was between the chicks fed the DAA-supplemented diet and those fed the high protein diet.

The expression pattern of the mRNA for malic enzyme and histidase was the same as it was for Experiment 4, except that histidase expression in the chicks fed the DAA-supplemented diet was intermediate between the expression levels found in chicks fed the basal and high protein diets. Representative Northern blots from Experiment 5 are shown in Figure 4.

DISCUSSION

Previous research has shown that an increase in the concentration of dietary protein or amino acids stimulates the activities of the regulatory enzymes of amino acid catabolism (Reynolds et al., 1971; Featherston and Horn, 1973; Miller et al., 1988; Ewart et al., 1992; Bella et al., 1996; Davis and Austic, 1997; Tovar et al., 2002; Chendrimada and Davis, 2005) and decreases the activity of malic enzyme, a key regulatory enzyme in de novo fatty acid synthesis in birds (Rosebrough and Steele, 1985, 1990; Rosebrough et al., 1988, 1996, 1999, 2002; Adams and Davis, 2001). In addition, previous research has indicated that glucagon may play a key role in the metabolic changes caused by increased amino acid intake. Plasma glucagon levels are increased when animals receive an intravenous infusion of amino acids or an increased concentration of dietary protein (Ohneda et al., 1968; Kuhara et al., 1991; Minick et al., 1996; Tovar et al., 2002). In response to an amino acid load, glucagon also stimulates the clearance of excess amino acids in humans (Boden et al., 1990; Charlton et al., 1996). The current results provide further support that glucagon is one of the key regulatory factors that mediates the response of dietary protein intake on metabolism. Specifically, plasma glucagon concentrations were quickly elevated in chicks fed a high protein diet, and this increase in plasma glucagon was associated with subsequent changes in the mRNA expression of histidase and malic enzyme in these chicks. Furthermore, intravenous injections of glucagon increased the mRNA expression of histidase, the primary catabolic enzyme for histidine, and decreased the mRNA expression of malic enzyme, a key regulatory enzyme in de novo fatty acid synthesis in birds.

The rapid changes in histidase and malic enzyme mRNA expression seen in response to glucagon injections were not unexpected because glucagon regulates the mRNA expression of chicken hepatic malic enzyme (Lefevre et al., 1999) and rat hepatic histidase (Alemán et al., 1998; Torres et al., 1998; Tovar et al., 2002). The 5′ region of the chicken malic enzyme gene contains response elements for cAMP that could mediate the effect of glucagon on malic enzyme mRNA synthesis (Mounier, et al., 1997). A report on the human hepatic histidase gene also indicates that the promoter sequence has several binding sites for cAMP-responsive transcription factors (Suchi et al.,

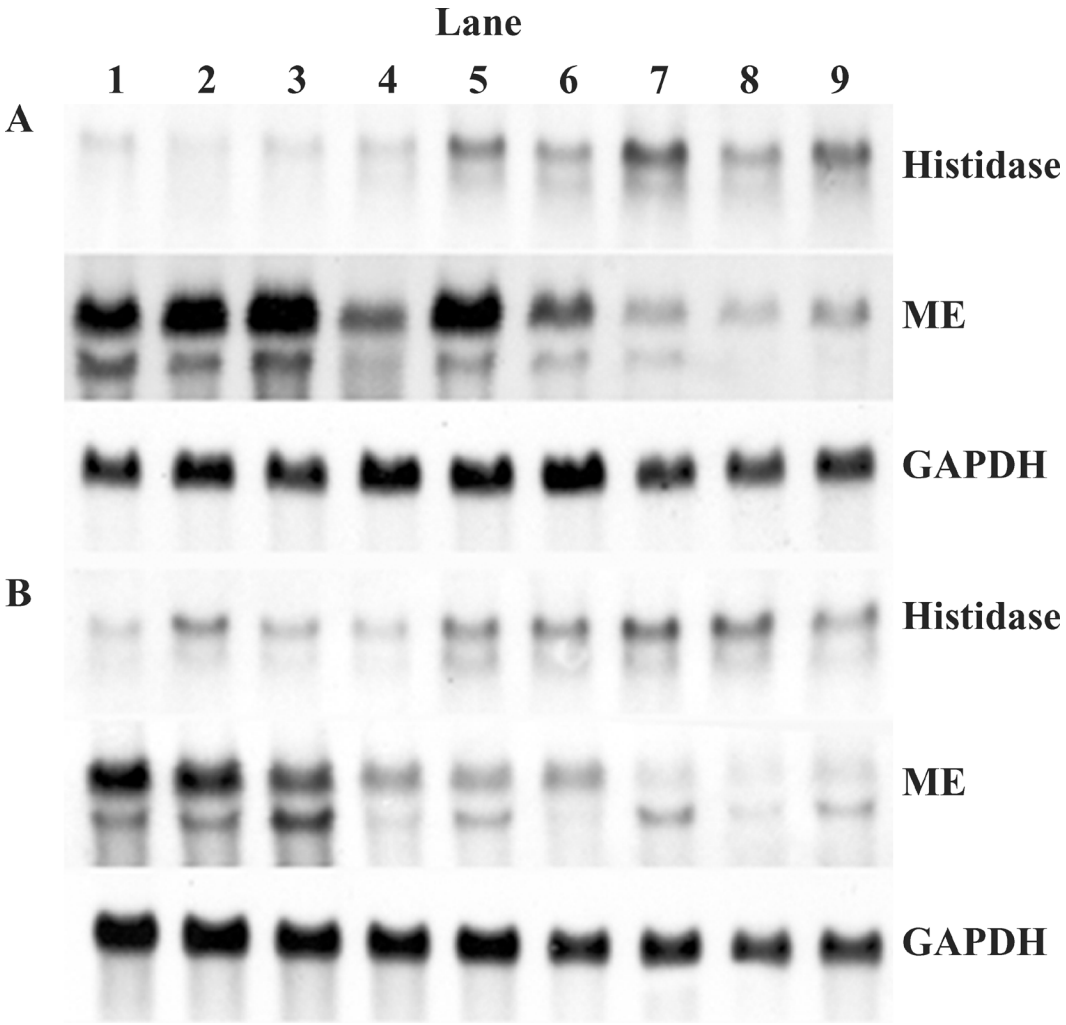

Figure 4. Autoradiograms from the Northern analysis of liver histidase and malic enzyme, showing 3 of the 6 replicate samples from each dietary treatment of the 24-h experimental duration (Experiment 5). Total RNA (40 μg) was loaded for each sample. Samples obtained from birds fed the basal diet, indispensable (A) or dispensable (B) amino acid-supplemented diet, and high protein diet are in lanes 1 to 3, 4 to 6, and 7 to 9, respectively. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; ME = malic enzyme.

1995), which would be necessary for the cAMP-mediated action of glucagon. Our results suggest that the chicken hepatic histidase gene promoter sequence also may have binding sites for cAMP-responsive transcription factors.

Comparing the plasma glucagon concentrations of chicks fed the basal protein diet or the high protein diet did not definitively establish that glucagon alone is responsible for altering the activities of malic enzyme and histidase when chicks consume elevated levels of dietary protein. After 1 h of feeding the 2 diets, chicks fed the high protein diet had a significantly higher plasma glucagon level than those fed the basal diet. A similar difference was observed at 3 h but not at 2 h. The lack of consistency in the results makes it difficult to assume a constant relationship between protein intake and plasma glucagon levels.

The birds were fasted 2 h before access was given to the basal and high protein diets to ensure immediate consumption of the diets. This fast would elevate plasma glucagon levels, and thus could explain why plasma glucagon levels were higher at the initiation of the experiment than after feeding for 1 h. The fast followed by the immediate consumption of food also may make the 1-h plasma glucagon values more reflective of the potential influence of dietary protein.

Glucagon secretion from the pancreas is affected by numerous factors in chickens, including stress (Freeman and Manning, 1976) and a circadian rhythm (Apfelbaum et al., 1972). An intracardiac injection of glucagon (200 μg/kg of BW) reduced total food consumption of chickens at 2 h but not at 1, 3, or 4 h after injection when compared with saline-injected control chickens (Smith and Bright-Taylor, 1974). Ideally the birds fed the basal and the high protein diets would have consumed the same amount of food in the present research. But, birds fed the high protein diet consumed less food than birds fed the basal diet. Hence, the difference in food consumption could be a confounding factor in the detected difference in plasma glucagon concentration obtained in the chicks fed the 2 diets.

Interestingly, specific amino acids have been implicated in promoting glucagon release and synthesis (Pipeleers

et al., 1985; Paul et al., 1998). Furthermore, research in chicks indicates that increased dietary sulfur amino acid intake decreases the activity of malic enzyme (Takahashi and Akiba, 1996) and that the addition of lysine or sulfur amino acids to the diet modifies lipid metabolism (Yeh and Leveille, 1969; Rosebrough et al., 1986; Mendonca and Jensen, 1989; Pesti et al., 1996). The results obtained from supplementing the basal diet with mixtures of DAA or IAA, however, indicate that if malic enzyme and histidase mRNA expression are regulated by specific amino acids, then it would have to be a mixture of specific indispensable and dispensable amino acids because the DAA and the IAA mixtures failed to elicit a response equivalent to feeding the high protein diet. Of course, another interpretation of these results could be that the regulation of the mRNA expression of these 2 enzymes may be due to the total nitrogen content of the diet. The total nitrogen content of the basal, high, IAA, and DAA diets was 2.88, 5.23, 4.45, and 3.91 (g/100 g of diet), respectively. Therefore, it may not be surprising that the level of malic enzyme and histidase mRNA expression was also intermediate for chicks fed the basal diet supplemented with either DAA or IAA compared with those fed the basal or high protein diets. Further research is needed to determine if dietary supplements of individual amino acids or nonprotein nitrogen will elicit a change in both histidase and malic enzyme mRNA expression.

In summary, glucagon regulates malic enzyme and histidase mRNA expression in the chick, and it may mediate the rapid effects of increased dietary protein intake on malic enzyme and histidase mRNA expression. Mixtures of indispensable and dispensable amino acids are equally effective in altering the mRNA expression of malic enzyme and histidase in the chick, which may indicate that total dietary nitrogen intake is what regulates the expression of these 2 enzymes in the chick rather than specific amino acids.

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