Is There Peripheral or Ovarian Insulin Action Alteration in Broiler Breeder Hens Fed ad Libitum?

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ABSTRACT We investigated whether a change in peripheral glucose homeostasis, a local change in the insulin-related ovarian regulatory system, or both occurred in ad libitum-fed broiler breeder hens compared with feed-restricted counterparts. Feed-restricted (R, from 5 to 16 wk of age) and ad libitum-fed (A) hens from a standard commercial line (S) and an experimental dwarf genotype (E) were studied. Basal and stimulated plasma insulin and glucose concentrations were measured during the prebreeding and laying periods. In the basal state (after 16 h fasting) plasma glucose concentrations were significantly lower in SA chickens (−5% at 17 wk, −7.5% at 32 wk) compared with EA, SR, and ER chickens, with no difference in plasma insulin concentrations (n = 16). In 17-wk-old SA birds, 30 min after oral glucose loading, plasma glucose concentrations increased significantly compared with the basal state and were also significantly lower as compared with SR but did not differ significantly from EA and ER. Plasma insulin concentrations did not differ significantly between genotypes or regimens (n = 16). A potential modification of intracellular mediators involved in the regulation of cell growth and survival in small follicles that were overrecruited in SA compared with SR was also investigated in SA and SR hens at 32 wk. There was no effect of food restriction in phospho-Akt, Akt, phospho-ERK, and phospho-S6 in the small white ovarian follicles (n = 6) in the basal state and after 30 min of refeeding. In conclusion, the present study does not demonstrate any evidence of glucose intolerance during the prebreeding period, specific change in the ovarian small follicle insulin signalling pathway, or both, in laying broiler breeders fed ad libitum compared with feed-restricted hens.

Key words: broiler breeder, feed restriction, peripheral and ovarian insulin regulation

INTRODUCTION Changes in ovarian function and fattening are adverse consequences of ad libitum feeding in broiler breeder hens selected for rapid growth (Hocking et al., 1987; Hocking et al., 2002). Food restriction limits these consequences (Hocking, 1987). There is significant evidence in mammals that, in addition to their regulatory role on body composition, metabolic hormones such as growth hormone, insulin, insulin-like growth factor (IGF)-I, and leptin have an important role in the control of ovarian follicle development and are probably important mediators of the effects of dietary intake or energy balance on mammal fertility (Monget and Martin, 1997; Diskin et al., 2003). It has been established over the last decade that the insulin-related ovarian regulatory system (insulin, insulin receptor, IGF-I, IGF-I receptor, IGF-binding protein, and IGF-proteases) participates in normal follicle development (Poretsky et al., 1999). Moreover, a number of reproductive abnormalities associated with increased body fat and glucose intolerance appear to result from changes in the insulin-related ovarian regulatory system, as observed in humans (Poretsky et al., 1999) and domestic animals (Diamanti-Kandarakis and Bergiele, 2001; Diskin et al., 2003; Hunter et al., 2004).

In previous studies Hocking et al. (1987) showed that the ovaries of broiler breeder hens fed ad libitum contained twice as many yellow follicles as laying hens at the onset of laying. The additional follicles result in multiple ovulation and poor egg production because of internal ovulation or through the production of soft-shelled, misshapen, and double-yolked eggs. However, in a recent study it was reported that the expression of IGF family mRNA in the granulosa and theca of mature yellow follicles did not reflect the wide variations in productivity (Heck et al., 2003). Hocking and Robertson (2000) observed a higher proportion of white follicles (in the range of 1.8 to 5 mm in diameter) in a selected restricted male line than in an ad libitum-fed control line, whereas selection did not change white follicle numbers in the female.
line. In addition, studies of standard broiler breeder hens fed ad libitum (SA) or feed-restricted from 2 wk of age (SR) indicated a significantly (P < 0.01) higher number of yellow follicles in SA hens (8.3 follicles/ovary) compared with SR (6.6 follicles/ovary) at 42 wk of age (Heck et al., 2004). However, in the same birds, the number of white follicles (30 to 1,000 mg) were even more numerous than yellow follicles in SA hens (41.8 follicles/ovary) compared with SR hens (28.8 follicles/ovary; P < 0.0002, S. Métayer, unpublished data). The mechanisms involved in the laying defects observed in ad libitum-fed hens might thus not only be linked to late maturation and development of yellow follicles but also to an already excessive recruitment of small white follicles, at least in some genotypes.

In the growing chicken a change in glucose-insulin balance is associated with metabolic imbalance linked to fattening (Rideau, 1997). A change in peripheral glucose homeostasis might thus occur in ad libitum-fed standard broiler breeder hens compared with feed-restricted counterparts. We measured basal and stimulated plasma glucose and insulin concentrations in the prebreeding stage. Two genotypes were compared, a standard broiler breeder (S) and a dwarf experimental genotype (E) showing greater tolerance of ad libitum feeding (Heck et al., 2004; Bruggeman et al., 2005). We also investigated the potential activation of the insulin signalling in small follicles in the ovaries of 32-wk-old standard broiler breeder hens. In fact a selective modification of insulin action on the ovary could explain a difference in the regulation of cell proliferation, differentiation, or growth of white follicles as suggested by the higher number of white follicles observed in SA compared with SR hens (S. Métayer, unpublished results). Among the mediators involved, we focused our attention on ribosomal protein S6, ERK, and Akt, which are kinases with critical roles in the regulation of proliferation, differentiation, growth, protein synthesis, and survival (Burginger and Coffer, 1995; Seger and Krebs, 1995; Dufner and Thomas, 1999; Johnson et al., 2001). These kinases as well as plasma glucose and insulin levels were measured in the basal state and 30 min after feeding in SA and SR hens.

**MATERIALS AND METHODS**

**Animal Housing and Sampling**

One-day-old (SE) and broiler breeder female chicks were obtained from a commercial hatchery (Hubbard, Lyon, France). The E genotype carries the recessive sex-linked dwarf gene (dw) resulting in lower body weight (−30%) compared with standard line birds and better breeding efficiency. This line was included in the study because it requires a reduced degree of feed restriction compared with standard breeders to maximize egg output (Heck et al., 2004; Bruggeman et al., 2005). Chickens were fed on a starter diet from 0 to 6 wk, a grower diet from 7 to 20 wk of age, and a breeder diet from 20 wk of age as previously described (Bruggeman et al., 2005). Hens were allocated the regimens in accordance with Hubbard nutritional recommendations. Half of the chickens (SR, ER) were restricted from 5 to 16 wk of age following the Hubbard handbook guidelines to match a similar reference body weight curve. The other half (SA, EA) was fed ad libitum.

Glucose-insulin balance was studied during the prebreeding (17 wk) periods. Sixteen average-weight hens per treatment and genotype (S, E) were sampled at 17 wk of age after overnight fasting (16 h, basal state) and 30 min after oral glucose loading following the overnight fast [2 g/kg of BW with a 50% glucose solution (wt/vol) administered in the crop by oral intubation]. Blood samples were collected into syringes containing a 1% heparin solution (wt/vol), cooled at 0°C, and centrifuged at 4°C. Plasma samples were separated, divided into aliquot samples, and stored at −20°C until assay. Fat pads were weighted after blood sampling on 6 hens per treatment (17 wk) and further checked at slaughter on 20 hens per treatment (40 wk).

For insulin signalling studies, average weight SA and SR hens (32 wk old) were assigned to the following nutritional states: food-deprived for 16 h or re-fed for 30 min after 16 h of food deprivation. Thirty min of re-feeding has been shown to be sufficient to induce kinase activation in chicken peripheral tissues (Bigot et al., 2003). After blood sampling (n = 16 hens per treatment) and euthanizing the birds by intravenous administration of an overdose of sodium pentobarbital, tissue was sampled on 6 hens per treatment. The abdominal cavity was opened, and the small (white) follicles (1 to 8 mm) diameter were excised from the ovary and stored at −80°C prior to analysis. All experiments were carried out with due regard to legislation governing the ethical treatment of animals, and investigators were authorized by the French government to carry out animal experiments.

**Analysis**

**Plasma Glucose and Insulin Concentrations.** Plasma glucose was determined with an automated method using the glucose oxidase method (Beckman glucose analyser). Plasma insulin was determined by radioimmunoassay as previously described (Simon et al., 1974) with a guinea pig antiporcine insulin serum (Ab 27-6), pure chicken insulin as standard, and 125I porcine insulin as tracer. The interassay coefficient of variation is 9%.

**Western-Blot Analysis.** Nitrocellulose membranes and the premade polyacrylamide solution were purchased from BioRad Laboratories (Hercules, CA). Anti-phospho-Ser473 Akt, antiAkt, antiphospho-Thr202/Tyr204 ERK, and antiphospho-Ser235/236 S6 were obtained from Cell Signaling Technology (Beverly, MA). Antitubulin was from Oncogene Research products (San Diego, CA). Tissue samples were solubilized in a buffer containing NaCl (150 mmol/L), tri(hydroxymethyl)-aminomethane (Tris) (pH 7.4; 10 mmol/L), EDTA (1 mmol/L), ethylene glycol-bis [b-aminoethyl ether]-N, N, N0, N0-tetra-acidic acid (1 mmol/L), triton X-100 (1%,
Table 1. Average BW and fat pad in standard (S) and experimental (E) broiler breeder hens fed ad libitum (A) or restricted (R) at 17 (n = 16 hens per treatment) and 40 wk of age (n = 20 hens per treatment of the basal state) 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SA</th>
<th>SR</th>
<th>EA</th>
<th>ER</th>
<th>g</th>
<th>r</th>
<th>g x r</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>4,340</td>
<td>1,770</td>
<td>2,752</td>
<td>1,626</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>38</td>
</tr>
<tr>
<td>Fat pad (%)</td>
<td>6.3a</td>
<td>0.6a</td>
<td>3.5b</td>
<td>1.1c</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.3</td>
</tr>
</tbody>
</table>

| Body weight (g) | 4,928 | 3,765 | 3,530 | 2,944 | 0.001 | 0.001 | 0.001 | 48  |
| Fat pad (%) | 5.17a | 3.56b | 3.63b | 2.98b | 0.001 | 0.01  | 0.10  | 0.23 |

**ANOVA (P <)**

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*Means within a line lacking a common superscript differ significantly (P < 0.05).

*Data were analyzed using 2-way ANOVA; g = genotype effect; r = regimen effect.

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Statistics

The values given are means ± SE. For the glucose and insulin concentrations, given the obvious wide variations between stages, comparisons for each (basal and refed) stage separately were performed using 2-way ANOVA with the genotype and the feed allowance levels as main effects (Statview Software program, version 5, Abacus Concepts Inc., Berkeley, CA). For the white follicles, Western blot results were compared by a 2-way ANOVA with the feed allowance levels (SA or SR) and the feeding stage (basal or refed) as main effects. A P-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In this study we investigated whether a change in peripheral glucose homeostasis, an ovary change in the insulin-related ovarian regulatory system, or both occurred in ad libitum-fed (SA and EA) broiler breeder hens compared with counterparts feed-restricted during the growing period (SR and ER).

The SA hens were significantly heavier than their restricted counterparts (SR) at 17 and 40 wk (Table 1). They were also heavier than the E genotype hens. Feed restriction also significantly reduced BW in 17 and 40 wk E hens (P < 0.05, Table 1), although the reduction was significantly less for the E than for the S genotype (significant interaction, P < 0.05) in spite of a similar feed intake to BW ratio from 5 to 16 wk of age (not shown). Although feed restriction decreased fattening at 17 and 40 wk for both genotypes (P < 0.05, Table 1), the relative fat pad weight at 40 wk of age did not differ among SR, ER, and EA.

Basal plasma glucose concentration did not differ among SR, EA, and ER at 17 wk of age, but it was significantly lower (−5%) in SA hens compared with SR, EA, and ER (P < 0.05, Table 2). Basal plasma insulin did not differ between regimens and genotypes. Thirty minutes after oral glucose loading, plasma glucose concentrations were higher than in the fasted state (P < 0.05, not shown on Table 2) and lower in SA birds compared with SR birds (P < 0.05); it did not differ significantly between EA and ER hens. Concomitantly, plasma insulin concentration increased significantly in response to the glucose loading (P < 0.05, not shown in Table 2), but levels did not significantly differ among regimens and genotypes. Basal plasma insulin concentration is considered a marker of insulin resistance in mammals (Monzillo and Hamdy, 2003). Plasma insulin and glucose concentrations measured after a glucose challenge are indicative of pancreatic and tissue responsiveness. The present findings showing no significant difference in basal insulin concentration between ad libitum fed (SA) and feed-restricted (SR) hens suggest that there is no peripheral insulin resistance induced by ad libitum feeding from hatching until onset of laying as compared with a restriction program of 11 wk during the prebreeding period. In contrast, the significantly lower plasma glucose concentrations observed in the basal state and 30 min after glucose loading observed in SA, without differences in plasma insulin concentrations, might result from better insulin sensitivity, thus leading to higher glucose utilization, as suggested by the heavier BW and fat pad observed in SA compared...
with SR and E birds. The present results are in accordance with observations on fat and lean line chickens (Touchburn et al., 1981), although this model is quite different on a genetic basis with ad libitum and restricted broiler breeders. Fat chickens in both the fasted and fed states exhibited slight hypoglycaemia, with similar (or slightly higher) insulin concentrations to lean chickens. Fat chickens appeared slightly more sensitive to exogenous insulin in the fasted state (Saadoun et al., 1988). In addition, significant activation of early stages of insulin signalling was observed in the livers of the fat line broilers (Dupont et al., 1999). When comparing BW, feed restriction appears relatively less drastic in the dwarf genotype (E) compared with the normal standard breeder hen (S), which might explain the lack of variation in glucose and insulin plasma concentrations in the EA genotype compared with ER.

In a second experiment we studied whether a selective modification of insulin sensitivity on the ovary could explain a difference in the regulation of cell proliferation, differentiation, or growth of white follicles. We therefore investigated the insulin signaling changes in the ovary by measuring the potential activation of Akt, S6, and ERK in laying standard hens in the basal and stimulated states as well as peripheral plasma glucose and insulin levels. At 32 wk of age, SA hens were significantly heavier than SR hens (5.271 ± 0.66 vs. 3.552 ± 0.44 g, mean ± SEM, n = 16, P < 0.05). Basal plasma glucose concentration was again significantly lower in SA hens compared with SR hens (9.8 ± 0.3 vs. 10.7 ± 0.2 mmoles/L, n = 16, P < 0.05) with no difference in the basal plasma insulin level (106 ± 16 vs. 105 ± 23 pmoles/L, n = 16, P > 0.05). During the 30 min refeeding test, SA birds ate significantly less than SR, (25 ± 2 vs. 39 ± 4 g/30 min, respectively, n = 6, P < 0.05). Thirty minutes after refeeding, plasma glucose and insulin levels increased significantly vs. basal levels (P < 0.05) but did not differ significantly between SA and SR (respectively 11.6 ± 0.3 vs. 12.3 ± 0.4 mmoles/L, n = 8, P > 0.05 for plasma glucose and 311 ± 56 vs. 395 ± 47 pmoles/L, n = 8, P > 0.05 for insulin levels). Phospho-Akt, Akt, phospho-ERK, and phospho-S6 levels did not differ between SA and SR hens (Table 3, Figure 1). When comparing the feeding state (refed vs. fasted), Akt, ERK, and S6 were not more phosphorylated (i.e., activated) after 30 min of refeeding in white follicles compared with the basal stage in SA and SR hens. We noted that SA and SR hens already presented a relatively high basal phosphorylated level in the fasted state. The findings thus did not show any difference in the phosphorylation of the kinases for any effect tested, i.e., regimen (ad libitum vs. restricted) or treatment (fasted vs. refed). These results do not support the hypothesis of a possible insulin-linked difference in the regulation of cell proliferation, differentiation, or growth of white follicles. This contrasts with peripheral basal plasma glucose and insulin levels, which suggest better insulin sensitivity in SA hens at 17 and 32

### Table 2. Plasma glucose and insulin concentrations in standard (S) and experimental (E) broiler breeder hens fed ad libitum (A) or restricted (R) in broiler breeder hens fed ad libitum at 17 wk of age (n = 16 hens per treatment)\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SA</th>
<th>SR</th>
<th>EA</th>
<th>ER</th>
<th>g</th>
<th>r</th>
<th>g × r</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mmoles/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>9.6</td>
<td>10.4</td>
<td>9.9</td>
<td>10.1</td>
<td>NS</td>
<td>0.001</td>
<td>0.003</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose 30 min</td>
<td>16.7</td>
<td>19.2</td>
<td>16.3</td>
<td>17.5</td>
<td>NS</td>
<td>0.025</td>
<td>NS</td>
<td>0.8</td>
</tr>
<tr>
<td>Plasma insulin (pmoles/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>193</td>
<td>153</td>
<td>153</td>
<td>125</td>
<td>0.06</td>
<td>0.06</td>
<td>NS</td>
<td>18</td>
</tr>
<tr>
<td>Glucose 30 min</td>
<td>261</td>
<td>303</td>
<td>270</td>
<td>246</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^1\)Means within a line lacking a common superscript differ significantly (P < 0.05).

### Table 3. Phosphorylation of intracellular kinases involved in insulin signalling in white follicles from standard broiler breeder hens fed ad libitum (SA) or feed-restricted (SR) at 17 wk of age (n = 16 hens per treatment)\(^1\)

<table>
<thead>
<tr>
<th>Regimen (r)</th>
<th>SA</th>
<th>Basal</th>
<th>Refed</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt/SA</td>
<td>0.94</td>
<td>1.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td>0.97</td>
<td>1.05</td>
<td>0.95</td>
<td>1.12</td>
</tr>
<tr>
<td>S6/Tubulin</td>
<td>0.91</td>
<td>1.18</td>
<td>0.97</td>
<td>1.03</td>
</tr>
<tr>
<td>ERK/Tubulin</td>
<td>1.35</td>
<td>0.74</td>
<td>0.97</td>
<td>1.12</td>
</tr>
<tr>
<td>Tubulin</td>
<td>8.30</td>
<td>8.52</td>
<td>8.80</td>
<td>7.72</td>
</tr>
</tbody>
</table>

\(^1\)NS = not significant (P > 0.05). Twenty-five micrograms of protein from white follicles (100 mg) were separated by 10% SDS-PAGE and immunoblotted using antiphospho-Akt, antiAkt, antiphospho-Erk1/2, antiphospho-S6, or anti α-tubulin. Bands were visualized and quantified by Odyssey LI-COR Biotechnology software (Lincoln, NE). Results of the phosphorylation of Akt, S6, and ERK were normalized for Akt and/or Tubulin content. The values are means with their pooled standard errors (SEM); n = 6. Data were analyzed using 2-way ANOVA; g = genotype effect; r = regimen effect, P-value was never inferior to 0.19.
Figure 1. Phosphorylation of Akt, S6, and ERK in white follicles from Standard broiler breeder hens. White follicles were compared between fasted control (–) and stimulated (+) animals fed ad libitum (SA) or feed-restricted from 2 wk of age onward (SR). Twenty-five micrograms of protein from white follicles (100 mg) were separated by 10% SDS-PAGE and immunoblotted using antiphospho-Akt (A), αAkt (B), antiphospho-ERK (C), or antiphospho-S6 (D). All the blotting membranes were reprobed with an antitubulin antibody to normalize gel loading (E). Bands were visualized and quantified by Odyssey LI-COR Biotechnology software (Lincoln, NE). The results of quantification are shown in Table 3.

wk. A complete kinetics study following oral glucose loading, exogenous insulin injection, or both would confirm this hypothesis.

In conclusion, the present study does not support the existence of an insulin resistance phenomenon nor provide evidence on the intracellular mechanisms responsible for ovarian dysfunction in fast-growing broiler breeder hens fed ad libitum.

ACKNOWLEDGMENTS

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