ABSTRACT A radioreceptor assay that specifically measures the affinity and concentration of glucagon receptors on the surface of broiler adipocytes was used to determine whether down-regulation of glucagon receptors is involved in the mechanism whereby glucagon induces desensitization of its ability to acutely stimulate lipolysis. Adipocytes from abdominal fat of 42- to 49-d-old female broilers were preincubated in vitro with 0 to 8 nM glucagon for 0 to 24 h before removal of glucagon by washing and assessment of lipolysis during a subsequent 1 h incubation at 37 C and binding of ([125I]iodo-Tyr10)glucagon during an ensuing 6 h incubation at 12 C. In Experiment 1, pretreatment of adipocytes with 0 to 8 nM glucagon for 24 h reduced lipolysis 70% with a preincubation dose of glucagon (ED50) of 0.4 ± 0.04 nM, whereas glucagon binding was decreased 75% with an ED50 of 0.7 ± 0.1 nM. In Experiment 2, pretreatment of adipocytes with 5.4 nM glucagon for 0 to 2 h decreased lipolysis 60% with a time of preincubation with glucagon (T1/2) of < 1 min, whereas glucagon binding was reduced 40% with a T1/2 of 2 ± 0.6 min. In Experiment 3, detailed analysis of lipolysis and glucagon binding to adipocytes pretreated with 5.4 nM glucagon for 5 min, 2 h, or 24 h revealed that sensitivity of adipocytes to acute stimulation of lipolysis by glucagon and the number of glucagon receptors on the adipocyte surface were decreased by glucagon pretreatment. The assay concentration of glucagon (EC50) for glucagon stimulation of lipolysis, a measure of adipocyte sensitivity, increased 0.8-fold at 5 min, 1.6-fold at 2 h, and 4.9-fold at 24 h of preincubation. The number of low-affinity glucagon receptors decreased 12% at 5 min, 20% at 2 h, and 50% at 24 h of preincubation with glucagon. Throughout this study, decreased glucagon binding was closely associated with reduced lipolysis, implicating down-regulation of cell-surface glucagon receptors in the mechanism whereby glucagon induces desensitization of its ability to acutely stimulate lipolysis in broiler adipocytes.

(Key words: adipocyte, desensitization, down-regulation, glucagon, lipolysis)

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and Iyengar, 1988) hepatocytes incubated in vitro with glucagon or from membranes prepared from livers of rats infused with glucagon (Soman and Felig, 1978). In all cases, the reduced binding of glucagon resulted from a reduction in the number of glucagon binding sites rather than a reduction in the affinity of glucagon binding.

The role of glucagon binding in the mechanism whereby glucagon induces desensitization of its acute metabolic effects in adipocytes has not been investigated. Consequently, the objectives of the present study were: 1) to determine whether in vitro preincubation of broiler adipocytes with glucagon alters glucagon binding to cell-surface receptors; 2) to determine whether a change in binding of glucagon to glucagon receptors of 42- to 49-d-old female broilers (Ross x Avian) and whether a change in binding affinity, or both are responsible for an alteration in glucagon binding following preincubation of broiler adipocytes with glucagon.

**Materials and Methods**

**Preincubation of Adipocytes**

Adipocytes were isolated from abdominal adipose tissue of 42- to 49-d-old female broilers (Ross x Avian) and maintained in primary culture using a serum-free, Dulbecco's modified Eagle's medium (DMEM) as described in Oscar (1991) and Harden and Oscar (1993). At 48 h in culture, adipocytes (1% vol/vol) were preincubated with 0 to 8 nM glucagon for 0 to 24 h. Pretreatment was accomplished by extensively washing adipocytes with hormone-free DMEM (Oscar, 1992) to remove glucagon before assessment of lipolysis and glucagon binding. Adipocytes destined for lipolysis assays were washed thrice at 37 C, whereas adipocytes destined for glucagon binding assays were washed thrice at 12 C.

**Biochemical Assays**

Lipolysis assays were conducted as previously described (Oscar, 1991). In brief, pretreated adipocytes (3% vol/vol) were incubated for 1 h at 37 C in 300 μL of DMEM that contained 0 to 2.7 nM glucagon. Glycerol released into DMEM was used as the index of lipolysis and was measured using a commercial kit. Specific binding of glucagon to receptors on the surface of broiler adipocytes was assayed using the method of Oscar (1995). Briefly, pretreated adipocytes (3% vol/vol) were incubated for 6 h at 12 C in 300 μL of DMEM that contained 50 pM ([125I]iodo-Tyr10)glucagon6 and 0, 0.1, 0.5, 1, 5, 10, 50, 100, or 500 nM unlabeled glucagon. The amount of bound and free ([125I]iodo-Tyr10)glucagon at steady-state (i.e., 6 h) was determined by rapidly separating adipocytes from DMEM by centrifugation through silicon oil. Results were corrected for nonspecific binding, which was the amount of radioligand bound in the presence of 1.4 μM glucagon.

**Experimental Designs**

Each experiment was replicated four or five times with pretreatments applied to adipocytes from a different donor bird in each replicate. Experiment 1 was designed to determine the effect of preincubation dose of glucagon (ED50) on lipolysis and glucagon binding to broiler adipocytes and whether changes in lipolysis and glucagon binding are dose-related. Experiment 2 was designed to determine the effect of preincubation time with a maximally effective dose of glucagon on lipolysis and glucagon binding to broiler adipocytes and whether changes in lipolysis and glucagon binding were temporally related. Experiment 3, in addition to confirming a temporal relationship between changes in lipolysis and glucagon binding, was designed to determine whether glucagon pretreatment alters sensitivity (i.e., altered assay concentration of glucagon (EC50) of adipocytes to the acute lipolytic action of glucagon and whether temporal changes in glucagon binding resulted from a change in the number of glucagon binding sites, a change in binding affinity, or both.

In Experiment 1, adipocytes (five replicate experiments) were preincubated with 0, 0.27, 1.4, 2.7, 5.4, and 8 nM glucagon for 24 h before they were assayed for lipolysis in the presence of 0, 0.27, or 2.7 nM glucagon and specific binding of 50 pM ([125I]iodo-Tyr10)glucagon. In addition, adipocytes from three of the replicate experiments were fixed in osmium and electronically counted and sized (Oscar, 1995) to determine the effect of glucagon pretreatment on the size and number of adipocytes used in the assays.

In Experiment 2, adipocytes (four replicate experiments) were pretreated with 5.4 nM glucagon for 0, 1, 5, 10, 20, 30, 60, 120, or 1,440 (24 h) min before measurement of lipolysis in the presence of 0 or 0.27 nM glucagon and specific binding of 50 pM ([125I]iodo-Tyr10)glucagon. An untreated control was not included at each time of preincubation because we have repeatedly (Oscar, 1992, 1993, 1995, 1996; Suniga and Oscar, 1994) shown that between 48 and 96 h of culture, lipolysis and glucagon binding to control adipocytes do not change.

In Experiment 3, adipocytes (four replicate experiments) were preincubated with 5.4 nM glucagon for 0, 5, 120, or 1,440 (24 h) min. Full dose-response curves for

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3The volume concentration of adipocytes (i.e., percentage vol/vol) was determined by centrifugation in microhematocrit capillary tubes (Oscar, 1991).
5Boehringer Mannheim Biochemicals, Indianapolis, IN 46200.
6Amersham, Arlington Heights, IL 60005.
glucagon binding (Figure 1B) in a dose-response manner. Basal lipolysis was reduced 30% with an ED50 of 0.4 ± 0.1 nM, lipolysis in the presence of 0.27 nM glucagon was decreased 70% with an ED50 of 0.4 ± 0.04 nM, and lipolysis in the presence of 2.7 nM glucagon was reduced 55% with an ED50 of 0.5 ± 0.1 nM. Specific binding of ([125I]iodo-Tyr10)glucagon to adipocytes was reduced 75% with an ED50 of 0.7 ± 0.1 nM. Maximal reductions of lipolysis and ([125I]iodo-Tyr10)glucagon binding were observed at preincubation doses of 2.7 to 8 nM.

Persistent activation of lipolysis with glucagon reduced the mean diameter of adipocytes used to assess lipolysis and ([125I]iodo-Tyr10)glucagon binding by 7 to 15% but did not alter the number of adipocytes per assay tube (Table 1). Because the method used to count adipocytes was not sensitive enough to consistently detect differences between glucagon pretreatments, assay results were not expressed on an adipocyte number basis but rather were expressed on an adipocyte volume (i.e., 3% vol/vol) basis throughout this study.

**Experiment 2**

When adipocytes were preincubated with 5.4 nM glucagon, decreases (P < 0.05) in lipolysis (Figure 2A) and ([125I]iodo-Tyr10)glucagon binding (Figure 2B) occurred rapidly. Basal lipolysis decreased 70% with a T1/2 < 1 min, lipolysis in the presence of 0.27 nM glucagon (< 2 h of pretreatment) was reduced 60% with a T1/2 < 1 min, and ([125I]iodo-Tyr10)glucagon binding was decreased 40% with a T1/2 of 2 ± 0.6 min. The reduction of basal lipolysis was maximal by 5 min of pretreatment, whereas lipolysis...
in the presence of 0.27 nM glucagon and (\[^{125}\text{I}]\text{iodo-Tyr}^{10}\)glucagon binding decreased (P < 0.05) between 2 and 24 h of preincubation.

**Experiment 3**

Analysis of full-dose response curves for glucagon stimulation of lipolysis indicated that pretreatment with 5.4 nM glucagon reduced (P < 0.05) the set-point of lipolysis and adipocyte sensitivity to glucagon in a time-dependent manner (Figure 3). Basal lipolysis was reduced maximally (50%) at 5 min of preincubation. The EC\textsubscript{50} for glucagon stimulation of lipolysis, a measure of adipocyte sensitivity, increased 0.8-fold at 5 min, 1.6-fold at 2 h, and 4.9-fold at 24 h of preincubation (Figure 3 inset). Maximal lipolysis was reduced 25% at 5 min, 40% at 2 h, and 60% at 24 h of pretreatment.

Scatchard plots of glucagon binding to adipocytes were curvilinear (Figure 4) and were interpreted in terms of two binding sites: a high-affinity, low-capacity site and a low-affinity, high-capacity site. Conclusions about the effect of glucagon pretreatment on K\text{d} and B\text{max} of the high-affinity site were not made because of the high SEM for its binding parameters, especially at 24 h of preincubation. On the other hand, B\text{max} of the low-affinity site was decreased (P < 0.05) 12% at 5 min (Figure 4B), 27% at 2 h (Figure 4C), and 54% at 24 h (Figure 4D) of pretreatment with glucagon, whereas K\text{d} of this site was not altered by glucagon pretreatment.

**DISCUSSION**

In a previous study (Oscar, 1992), it was demonstrated that preincubation of broiler adipocytes with glucagon induces a dose-response and time-dependent desensitization of lipolysis stimulation by glucagon. The present study extended these earlier findings by more precisely defining the dose-response and time course effects of glucagon pretreatment on desensitization of lipolysis.

<table>
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<th>TABLE 1. Effect of preincubation dose of glucagon on mean diameter and number of adipocytes used to assess lipolysis and glucagon binding, Experiment 1$^1$</th>
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<tr>
<td><strong>Preincubation dose of glucagon</strong></td>
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<td>Mean adipocyte diameter, (\mu\text{m})</td>
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<td>Number of adipocytes per 3% vol/vol</td>
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$^1$Adipocytes were preincubated with the indicated doses of glucagon for 24 h. Data are from three replicates in which pretreatments were applied to adipocytes from a different donor bird in each replicate.

$^2$Probability for no effect of preincubation dose of glucagon.
FIGURE 3. Effect of time of preincubation with glucagon on full dose-response curves for glucagon stimulation of lipolysis in broiler adipocytes. Adipocytes were pretreated with 5.4 nM glucagon for 0 (●), 5 min (▲), 2 h (▼), or 24 h (▲). Lipolysis was assessed during an ensuing 1 h incubation at 37 C by exposing 3% vol/vol adipocytes to the indicated doses of glucagon. The EC₅₀ (inset graph) for glucagon stimulation of lipolysis were 55 ± 15 pM at 0 min, 98 ± 10 pM at 5 min, 143 ± 23 pM at 2 h, and 324 ± 36 pM at 24 h of preincubation. Each point is the mean ± SEM of four replicate experiments in which the pretreatment was applied to adipocytes from a different donor bird in each replicate.

Lipolysis. In general, glucagon pretreatment induced desensitization of lipolysis with an ED₅₀ of 0.4 nM and a T₁/₂ of < 1 min. Furthermore, desensitization of lipolysis varied with dosage and time, and was associated with reduced binding of glucagon to receptors on the adipocyte surface. Glucagon pretreatment reduced glucagon binding from 40 to 75% with an ED₅₀ of 0.7 nM and a T₁/₂ of 2 min.

Similar to our results, exogenous administration of glucagon to rats causes parallel reductions in glucagon activation of adenylyl cyclase and glucagon binding to liver cell preparations. Srikant et al. (1977) reported that twice daily injections of glucagon for 10 d reduced glucagon binding to liver membranes by 65%, whereas the ability of glucagon to stimulate adenylyl cyclase was decreased 20%. Soman and Felig (1978) found that after 5 h of glucagon infusion, binding of glucagon to and the ability of glucagon to stimulate adenylyl cyclase in rat liver membranes were decreased 45%. Santos and Blazquez (1982) reported that glucagon binding to hepatocytes from rats treated with glucagon every 8 h for 4 d was reduced 35%.

In vitro treatment of rat hepatocytes with glucagon also causes parallel reductions in glucagon activation of adenylyl cyclase and glucagon binding to liver cell preparations. Premont and Iyengar (1988) found that glucagon binding to membranes from chicken hepatocytes was reduced maximally (55%) after 5 min of incubation with 1 μM glucagon. At 30 min of incubation, glucagon binding was reduced maximally by 300 nM glucagon with an ED₅₀ between 1 and 30 nM. Noda et al. (1984) showed that glucagon binding to membranes from rat hepatocytes incubated with 0.5 nM glucagon was reduced maximally (65%) between 6 and 12 h of incubation with a T₁/₂ of 2 h. The ability of glucagon to stimulate cyclic adenosine monophosphate production from hepatocyte membranes was decreased 65% after 9 h of incubation with 0.5 μM glucagon. Santos and Blazquez (1982) reported that glucagon binding to hepatocytes was reduced 10, 25, and 45% when hepatocytes were incubated for 6 h with 1, 5.7, and 28 nM glucagon, respectively. Significant reductions in glucagon binding were noted at 6 and 4 h but not at 2 h of incubation with 28 nM glucagon. Thus, similar to our results, exposure of rat liver cells to elevated levels of glucagon either in vivo or in vitro reduces glucagon binding by less than 75%. In addition, decreased glucagon binding to glucagon-treated liver cell preparations varies with dosage and time and is associated with desensitization of glucagon's acute metabolic effects.

Decreased binding of glucagon to cell preparations previously exposed to glucagon may result from
FIGURE 4. Scatchard analysis of the effect of time of preincubation with glucagon on glucagon binding to broiler adipocytes. Adipocytes were pretreated with 5.4 nM glucagon for 0 min (panel A), 5 min (panel B), 2 h (panel C), or 24 h (panel D). After preincubation, glucagon binding was assessed by incubating 3% vol/vol adipocytes for 6 h at 12 C with 50 pM ([125I]iodo-Tyr10)glucagon and 0 to 500 nM unlabeled glucagon. Results were corrected for nonspecific binding, which was the amount of radioligand bound in the presence of 1.4 uM unlabeled glucagon. Each point is the mean ± SEM of four replicate experiments in which the pretreatment was applied to adipocytes from a different donor bird in each replicate. Steady-state binding affinity (Kd) and binding capacity (Bmax) are presented for glucagon receptors in the high-affinity (Site 1) and low-affinity (Site 2) states.

decreased numbers of binding sites (i.e., down-regulation of receptors), decreased binding affinity, or both. In Experiment 3 of this study it was demonstrated that preincubation of broiler adipocytes with glucagon reduced binding capacity, a measure of glucagon receptor numbers, but did not alter binding affinity of low-affinity glucagon receptors. A similar trend was noted for high-affinity glucagon receptors but the high SEM obtained for the high-affinity binding site parameters, especially for adipocytes pretreated with glucagon for 24 h, rendered the results inconclusive. Nonetheless, in a previous study (Oscar, 1995), we found that high-affinity glucagon receptors account for only 2% of the total complement of glucagon receptors on the surface of broiler adipocytes. High-affinity receptors also appeared to represent less than 10% of the complement of cell-surface glucagon receptors in the present study (Figure 4). Therefore, we conclude that the
reduced binding of glucagon to adipocytes pretreated with glucagon was due to down-regulation of glucagon receptors on the surface of broiler adipocytes. Moreover, down-regulation of glucagon receptors was dose-related and temporally associated with desensitization of lipolysis throughout this study, indicating that down-regulation of glucagon receptors is involved in the mechanism whereby glucagon induces desensitization of its ability to acutely stimulate lipolysis. Consistent with our conclusions, decreased binding of glucagon to liver cell preparations exposed to elevated levels of glucagon in vivo (Srikant et al., 1977; Soman and Felig, 1978) or in vitro (Santos and Blazquez, 1982; Noda et al., 1984; Premont and Iyengar, 1988) also results from down-regulation of glucagon receptors rather than decreased binding affinity.

Down-regulation of hormone receptors coupled to G proteins involves three steps (Sibley et al., 1986). First, within seconds to minutes after binding ligand, G protein-coupled receptors are phosphorylated by cellular kinases, uncoupling them from Gs and reducing their affinity for ligand. Second, phosphorylated receptors are rapidly (T 1/2 < 3 min) sequestered within the cell, resulting in down-regulation of cell-surface receptors. Third, within a few hours after ligand binding, internalized receptors are shunted to a degradative pathway, resulting in down-regulation of their total numbers in target cells. Although our assay only measures glucagon binding to receptors on the adipocyte surface, and therefore, is unable to distinguish between sequestered and down-regulated receptors, the rapid time course for down-regulation of cell-surface glucagon receptors (T 1/2 = 2 min) in this study is consistent with the rapid time course for sequestration of other G protein-coupled receptors. Furthermore, Premont and Iyengar (1988) reported that dissociation of glucagon from chicken hepatic membranes pretreated with glucagon for 5 min was faster than dissociation of glucagon from membranes of control hepatocytes. The faster rate of dissociation from pretreated membranes suggests that a greater proportion of receptors from pretreated cells were in the low-affinity or uncoupled state. Thus, down-regulation of glucagon receptors appears to occur by a mechanism that is similar to the mechanism for down-regulation of other G protein-coupled receptors (Sibley et al., 1986).

The ability of 5.4 nM glucagon to reduce glucagon binding at 24 h of preincubation was variable between batches of adipocytes. For the 13 batches of adipocytes, each from an individual donor bird, used in the experiments illustrated in Figures 1B, 2B, and 4, the mean reduction of glucagon binding was 58.7 ± 3.62% (mean ± SEM) with a range from 42.9 to 84.0%. Although experimental protocols were standardized between batches of adipocytes, subtle changes in culture ingredients, glucagon solutions, and the physiology of donor birds may account for the observed variability in pretreatment effects on glucagon binding.

In summary, preincubation of broiler adipocytes with glucagon induced a dose-response and time-dependent reduction of lipolysis and glucagon binding to cell-surface receptors. The decreased binding of glucagon resulted from a decrease in glucagon receptor numbers rather than from a decrease in binding affinity. Throughout this study, decreased glucagon binding was closely associated with reduced lipolysis, implicating down-regulation of cell-surface glucagon receptors in the mechanism whereby glucagon induces desensitization of its ability to acutely stimulate lipolysis in broiler adipocytes.

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