Factors Affecting Insulin Responsiveness of Triglyceride Synthesis in Incubated Chicken Hepatocytes (43562)

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Abstract. **A technique was developed to isolate and incubate hepatocytes from 4-day-old chickens. Hepatocytes incubated in serum-free medium containing 1 ng chicken insulin/ml had greater rates of triglyceride (TG) synthesis than insulin-unexposed cells. Greater concentrations of insulin enhanced TG synthesis. Addition to medium of a supplement containing several hormones greatly improved insulin responsiveness of TG synthesis in hepatocytes compared with cells not incubated in supplemented medium. The medium supplement did not affect TG synthesis in hepatocytes incubated in the absence of insulin. The presence, compared with the absence, of triiodothyronine in the medium supplement improved insulin responsiveness of TG synthesis. In hepatocytes incubated in supplemented medium, increasing chicken insulin concentrations augmented malic enzyme activity. Hepatocytes incubated in supplemented medium containing bovine insulin had lesser rates of TG synthesis than cells incubated with similar concentrations of chicken insulin. To determine whether insulin responsiveness of TG synthesis is affected by age of chicken, hepatocytes from 4-, 11-, and 74-day-old chickens were incubated in supplemented medium. Responsiveness of TG synthesis to chicken insulin progressively decreased in hepatocytes from older chickens. Results indicate that hepatocytes isolated from 4-day-old chickens and incubated in supplemented medium are responsive to dilute insulin concentrations and may be useful to investigate the biochemical effects of this hormone.** [P.S.E.B.M. 1993, Vol 202]

In vivo investigations suggest an important role of insulin in regulating triglyceride (TG) synthesis in chickens (1–4). Since liver is the primary site of TG synthesis for adipose deposition in chickens (5–7), incubation of hepatocytes in a controlled and chemically defined environment could be a useful method to investigate the effect of insulin on TG synthesis.

Although incubated chicken hepatocytes have been used to investigate the effect of insulin on TG synthesis (8–10), results from such studies have been difficult to extrapolate to live chickens because of hepatocyte unresponsiveness to insulin at physiologic concentrations. For instance, consumption of a meal increases plasma insulin concentrations from 0.5 ng/ml to 4.0 and 9.5 ng/ml in lean and fat chickens, respectively (2). This observation suggests a relationship between plasma insulin concentrations and TG accretion in live chickens. In incubated chicken hepatocytes, however, acetate incorporation into total lipids increases at 100 ng insulin/ml but not at 10 ng/ml, compared with insulin-unexposed cells (8). Furthermore, acetate incorporation into fatty acids is unaffected by incubating chicken liver slices in medium supplemented to 50 ng insulin/ml (9).

Incubated hepatocyte unresponsiveness to plasma concentrations of insulin may be due to the absence of several hormones in incubation medium that cells are exposed to *in vivo*. Previous investigators studied the role of insulin on lipogenesis using incubated chicken hepatocytes or liver slices in chemically defined medium supplemented with few hormones (8–10). Hepatocyte incubation in medium supplemented with several hormones may be more relevant to the *in vivo* milieu of chickens and may improve hepatocyte insulin responsiveness.

To improve insulin responsiveness of TG synthesis in incubated hepatocytes, a simple technique was de-

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veloped to isolate hepatocytes from young posthatch chickens and incubate these cells in chemically defined medium supplemented with several hormones. We demonstrate responsiveness of hepatocyte TG synthesis to manipulations of insulin concentration that is within the range of plasma insulin concentrations reported for live chickens. We also report factors that affect insulin responsiveness of TG synthesis in these cells and discuss the usefulness of our method to investigate insulin-stimulated TG synthesis.

Materials and Methods

Hepatocyte Isolation and Incubation. Livers were extracted from 4-day-old female broiler chickens, rinsed in ice-cold Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} (Sigma Chemical Co., St. Louis, MO), and minced using scissors to 1-mm^3 pieces in 5 ml of HBSS containing 1 mg collagenase Type IV/ml (collagenase buffer; Sigma). The mince was diluted 10-fold with collagenase buffer and incubated in flasks for 1 hr in a water bath at 37°C. Separate flasks were used for livers extracted from different chickens. The mince was pipetted up and down every 10 min during incubation to accelerate hepatocyte dissociation.

After incubation, the digestion mixture was filtered through a 64-μm nylon screen and filtrate was centrifuged for 2 min at 250*g* and 4°C. Supernatant was discarded and pellets were quickly resuspended in ice-cold HBSS. Centrifugation was repeated three times to separate hepatocytes from erythrocytes. Upon completion of the final centrifugation, hepatocytes were resuspended in ice-cold Dulbecco's modified Eagle's/Ham's F12 medium (Sigma) supplemented to 0.2 ng putrescine/ml, 1 ng bovine insulin/ml, 1 ng bovine glucagon/ml, 1 ng mouse epidermal growth factor/ml, 1 ng corticosterone/ml, 1 ng human recombinant insulin-like growth factor-1/ml (Collaborative Research Inc., Bedford, MA), 1 ng bovine fibroblast growth factor/ml, 1 ng tripeptide Gly-His-Lys/ml, 1 ng purified chicken growth hormone (GH)/ml (Research and Education Institute, Torrance, CA), 3 ng triiodothyronine (T_3)/ml, 1 μg bovine transferrin/ml, 50 μg gentamicin sulfate/ml, 10% (v/v) chicken embryo extract (CEE; Gibco Laboratories, Grand Island, NY), and 5 m*M* acetic acid. Medium supplements, unless otherwise noted, were purchased from Sigma.

Hepatocytes isolated from different chickens were mixed. Hepatocyte number and viability were then determined by hemacytometer and trypan blue exclusion, respectively. Erythrocytes represented less than 5% of total cells counted and hepatocyte viability was greater than 90%. Hepatocyte number in medium was adjusted to 2.7×10^6 viable cells/ml and 2 ml were added to 35-mm diameter wells (Corning Glass Works, Corning, NY). At least nine wells of hepatocytes were obtained from each chicken liver.

Hepatocytes were incubated at 40°C and 5% (v/v) CO_2 in a humidified Steri-Cult 200 incubator (Forma Scientific, Inc., Marietta, OH). After 24 hr of incubation, cells were exposed to experimental treatments.

Effect of Simultaneous Addition of Several Hormones on Insulin Responsiveness of TG Synthesis. The effect of several purified hormones, simultaneously added to culture medium, on insulin responsiveness of TG synthesis was examined in hepatocytes incubated with different insulin concentrations. Thirty-six wells of hepatocytes, previously incubated for 24 hr, were washed with HBSS and replenished with Dulbecco's modified Eagle's/Ham's F12 medium. For 18 wells, medium was supplemented with 50 μg gentamicin sulfate/ml and 5 m*M* acetate (Medium 1). For the remaining wells, Medium 1 was further supplemented with putrescine, glucagon, epidermal growth factor, corticosterone, insulin-like growth factor-1, fibroblast growth factor, Gly-His-Lys, GH, T_3, and transferrin to concentrations used during the previous incubation period (Medium 2). CEE was not added to Medium 1 or 2. All wells of hepatocytes were then exposed to 0, 1, or 10 ng chicken insulin/ml (chicken insulin; gift from Dr. R. L. Hazelwood, University of Houston, Houston, TX). After 24 hr of incubation, cells were replenished with the previous medium (Medium 1 or 2) and TG synthesis was determined.

TG synthesis was determined by incubating three wells of hepatocytes for each treatment with [2-^{14}C] sodium acetate (2 μCi/well; 6.8 Ci/mole; ICN Biomedicals, Inc., Costa Mesa, CA). After 3 hr of incubation, radiolabeled medium was removed and hepatocytes were gently washed twice with ice-cold phosphate-buffered saline (pH 7.4). Hepatocytes were scraped from their wells and lipids extracted (11) by chloroform/methanol (2/1; v/v). Extract solvent was dried under a nitrogen stream and lipids were resolubilized in chloroform/methanol. Separation of lipids was accomplished by thin layer chromatography (12, 13). When thin layer chromatography plates were dried, lipid spots were visualized using iodine vapor and TG identified by comparison to triolein-TG standard (Sigma). TG spots were scraped from thin layer chromatography plates into scintillation vials and ^{14}C was quantified by scintillation spectrometry. Rate of [2-^{14}C]acetate incorporation into TG was linear (R^2 = 0.98) for 7.5 hr (unpublished results).

Measurements of TG synthesis were related to intracellular DNA quantity. DNA was determined fluorometrically (14) in hepatocytes not incubated with radiolabel (three wells/treatment) but subjected to the same incubation conditions as those incubated with radiolabel. DNA measurements in hepatocytes from different wells were averaged for each treatment.

Data were statistically analyzed by analysis of variance (SAS Institute, Cary, NC). Significant ($P < 0.05$) differences among treatment means were determined by least significant differences test.

Effect of Type of Insulin on TG Synthesis. Because of the scarcity of chicken insulin, the effect of chicken insulin on hepatocyte TG synthesis was compared with commercially available bovine insulin. Thirty-six wells of hepatocytes, previously incubated for 24 hr, were washed with HBSS and replenished with Medium 2. Chicken or bovine insulin were added to medium to 0, 1, or 10 ng/ml. After 24 hr of incubation, hepatocytes were replenished with previous medium and supplements. TG synthesis and statistical analyses of data were determined as described above.

Effect of T_3 on Insulin Responsiveness of TG Synthesis. The concentration of T_3, a documented regulator of lipogenesis (15–17), was manipulated in medium to determine effects on insulin responsiveness of hepatocyte TG synthesis. Seventy-two wells of hepatocytes, previously incubated for 24 hr, were washed with HBSS and replenished with Medium 2 adjusted to 0, 3, or 30 ng T_3/ml. Chicken insulin was then added to media to 0, 1, 10, or 50 ng/ml. After 24 hr of incubation, hepatocytes were replenished with the previous medium and supplements. TG synthesis and statistical analyses of data were determined as described above.

Determination of Insulin Responsiveness of TG Synthesis in Hepatocytes Isolated from Chickens of Different Ages. Hepatocytes were isolated simultaneously from 4-, 11-, and 74-day-old female chickens and incubated with different insulin concentrations to determine the effect of age of chicken on insulin responsiveness of TG synthesis. Livers were extracted from two chickens of each age, and hepatocytes were isolated and incubated as described previously. Eighteen wells of hepatocytes for each age of chicken were used in this experiment.

Hepatocytes, previously incubated for 24 hr, were washed with HBSS and replenished with Medium 2. Chicken insulin was added to medium to 0, 1, or 10 ng/ml. After 24 hr of incubation, hepatocytes were replenished with the previous medium and supplements. TG synthesis and statistical analyses of data were determined as described above.

Determination of Insulin Responsiveness of Hepatocyte Malic Enzyme Activity. Since malic enzyme is an important regulator of hepatic lipogenesis in chickens (16, 18, 19), the sensitivity of this enzyme to different insulin concentrations was determined in incubated hepatocytes. Eighteen wells of hepatocytes, previously incubated for 24 hr, were washed with HBSS and replenished with Medium 2. Chicken insulin was then added to medium to 0, 1, or 10 ng/ml. After 24 hr of incubation, hepatocytes were replenished with the previous medium and supplements. Hepatocytes were incubated for 3 hr, washed twice in ice-cold phosphate-buffered saline (pH 7.4), and scraped from their wells in 50 μl of phosphate-buffered saline. Cells from two wells were pooled to obtain one replicate, frozen, thawed, mixed using a pellet pestle (Kontes Scientific Instruments, Vineland, NJ), and centrifuged at 15,800g at 4°C for 30 min. Malic enzyme activity was determined by diluting 50 μl of supernatant 60-fold in 10 mM Tris hydroxymethyl aminomethane (pH 7.4), 45 μM NADP, and 0.5 mM malate. Conversion of NADP to NADPH was monitored fluorometrically at excitation and emission wavelengths 340 and 460 nm, respectively. Malic enzyme activities were related to protein quantity in cellular cytosols (20). Data were statistically analyzed as described above.

Results and Discussion

In vivo studies with chickens suggest that insulin regulates hepatic TG synthesis (1–3). Incubated chicken hepatocytes, however, require greater insulin concentrations than those measured in plasma to augment acetate incorporation into lipids (8). The responsiveness of incubated chicken hepatocytes to insulin must be improved so that conclusions from studies of TG synthesis in these cells can be confidently applied to live chickens.

The method we have developed to isolate hepatocytes from 4-day-old chickens does not require liver perfusion and can be accomplished in 4 hr when livers are extracted from six chickens. Figure 1 indicates that incubation of hepatocytes simultaneously with several hormones (Medium 2) improved insulin responsiveness of TG synthesis compared with cells incubated with insulin as the only hormone (Medium 1). Supplements added to medium did not affect TG synthesis in hepatocytes incubated in the absence of insulin. In Medium 1 or 2, TG synthesis in hepatocytes was augmented

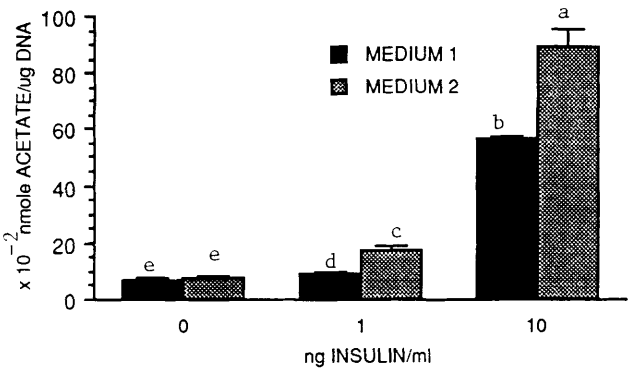

Figure 1. Effect of chicken insulin concentration on triglyceride synthesis in hepatocytes incubated in medium not supplemented (Medium 1) or supplemented (Medium 2) with several hormones. A different letter above each bar denotes a significant ($P < 0.05$) mean difference.

when increasing amounts of insulin were added to medium. Regardless of degree of hormone supplementation of medium, hepatocytes isolated using the described method were responsive to changes of insulin concentration. The insulin concentrations tested were within the range of insulin concentrations reported in live chickens (2).

An effort was made to avoid the use of CEE in medium during the measurement of TG synthesis. Omission of CEE in medium enabled precise manipulation of hormone concentrations in medium that would not have been possible had CEE been present. Hepatocytes were incubated with CEE for 24 hr after isolation because its presence in medium improved monolayer formation. Incubation of hepatocytes as a monolayer permits intercellular contact, which may be important for expression of hepatic functions (21).

Medium 2 contained several hormones in an attempt to represent the endocrine component of the extracellular milieu of the chicken. We believed that under these incubation conditions, the responsiveness of hepatocytes to plasma insulin concentrations would be more representative of *in vivo* responsiveness. Concentrations in Medium 2 of glucagon, corticosterone, and T_3 were at levels similar to those measured in chicken plasma (22). The remaining hormones in Medium 2 were added to dilute levels because of cost considerations or because chicken plasma concentrations were not well defined.

Because Medium 2 improved insulin responsiveness of TG synthesis (Fig. 1), experiments were designed to more precisely identify the hormones that were responsible for this effect. Increasing T_3 concentrations improved insulin responsiveness of TG synthesis (Table I). Compared with hepatocytes incubated in the absence of T_3, addition of T_3 to 3 ng/ml increased TG synthesis at any insulin concentration. Since the concentration of T_3 in chicken plasma is 3 ng/ml (22), our results suggest that plasma concentrations of T_3 and insulin have an important role in regulating TG synthesis in the chicken hepatocyte. In an experiment similar to that done with T_3, GH concentrations in Medium 2 also were manipulated. Increasing GH from 0 to 10 ng/ml decreased TG synthesis in hepatocytes incubated with 50 ng insulin/ml, but not in cells incubated in more dilute insulin concentrations (unpublished data). The effect of other hormones in Medium 2 on TG synthesis requires further investigation.

Because of the scarcity of suppliers of chicken insulin, the effect of chicken insulin on TG synthesis was compared with bovine insulin. Chicken insulin had a greater effect on TG synthesis compared with similar concentrations of bovine insulin (Fig. 2). Each increase in bovine insulin concentration, however, further augmented TG synthesis in incubated hepatocytes. These results suggest that bovine insulin can be an alternative to chicken insulin.

Age of the chicken used as a source of hepatocytes was a critical determinant of the responsiveness of TG synthesis to insulin (Table II). Either TG synthesis was less responsive to insulin or insulin's biologic effects were inhibited in incubated hepatocytes from older

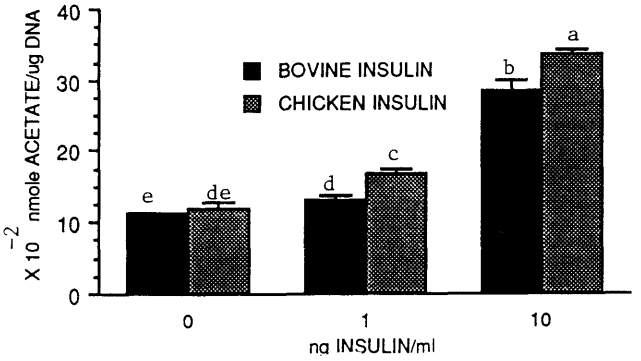

Figure 2. Comparison of bovine and chicken insulin effects on triglyceride synthesis in hepatocytes incubated in Medium 2. A different letter above each bar denotes a significant ($P < 0.05$) mean difference.

Table I. Triiodothyronine and Chicken Insulin Effects on Triglyceride Synthesis in Incubated Chicken Hepatocytes

Insulin (ng/ml)	Triiodothyronine		
	0 ng/ml	3 ng/ml	30 ng/ml
	($\times 10^{-2}$ *nmol acetate/μg DNA*)		
0	$6.4^{k} \pm 0.2$	$17.5^{j} \pm 0.3$	$22.2^{i} \pm 0.2$
1	$17.0^{j} \pm 0.2$	$32.4^{h} \pm 0.3$	$44.4^{g} \pm 0.6$
10	$88.1^{f} \pm 2.3$	$264.5^{e} \pm 6.2$	$294.5^{d} \pm 10.0$
50	$375.1^{c} \pm 9.2$	$644.7^{a} \pm 7.4$	$580.4^{b} \pm 19.9$

Note. Hepatocytes were incubated in Medium 2 containing [2-^{14}C] acetate but no triiodothyronine. Triiodothyronine and insulin were added to medium to the indicated concentrations. After 3 hr of incubation, triglyceride synthesis was determined as described in Materials and Methods. Means ± SE within the same row or column that have different superscripts (a–k) are significantly ($P < 0.05$) different.

Table II. Effect of Chicken Insulin on Triglyceride Synthesis in Incubated Hepatocytes from Chickens of Different Ages

Insulin (ng/ml)	Age of chicken		
	4 days	11 days	74 days
	($\times 10^{-2}$ *nmol acetate/μg DNA*)		
0	$7.8^{c} \pm 0.6$	$1.4^{e} \pm 0.1$	$0.2^{f} \pm 0.2$
1	$10.7^{b} \pm 0.6$	$1.6^{e} \pm 0.1$	$0.1^{f} \pm 0.1$
10	$55.1^{a} \pm 7.6$	$2.9^{d} \pm 0.1$	$0.1^{f} \pm 0.1$

Note. Hepatocytes from chickens of different ages were incubated for 3 hr in Medium 2 containing [2-^{14}C]acetate and the indicated concentration of insulin. After the incubation period, triglyceride synthesis was determined as described in Materials and Methods. Means ± SE within the same row or column that have different superscripts (a–f) are significantly ($P < 0.05$) different.

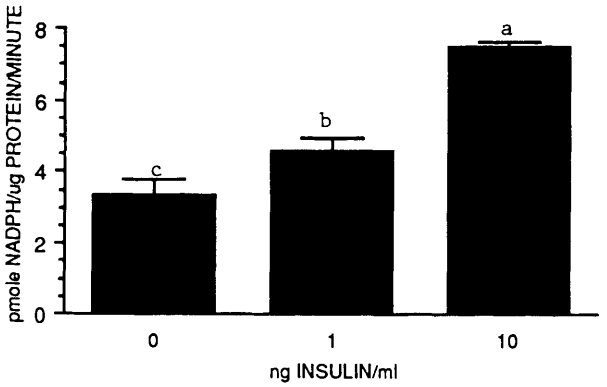

Figure 3. Effect of chicken insulin concentration on malic enzyme activity in hepatocytes incubated in Medium 2. A different letter above each bar denotes a significant ($P < 0.05$) mean difference.

rather than younger chickens. Perhaps adjustments of the type and concentration of supplements added to medium are required to improve insulin responsiveness of TG synthesis in hepatocytes from older chickens.

Differences in TG synthesis among endocrine treatments in our studies were not due to fluctuations of intracellular acetate specific activity (μCi/mmol). Similar results were obtained whether tritiated water or [2-^{14}C]acetate was used as the radiolabel (unpublished results). Furthermore, incubation of hepatocytes with increasing chicken insulin concentrations augmented malic enzyme activity (Fig. 3).

Attempts to decrease fat accretion without growth reduction in chickens fed or injected with hormones have been disappointing (23, 24). More research of the endocrine regulation of TG synthesis in the chicken is required before mechanisms controlling fat accretion can be understood. Chicken hepatocytes incubated under conditions described in this study can be a useful tool to investigate interactions of insulin with other hormones, insulin-receptor binding, and insulin-signal transduction. This study shows that defined media can be developed to yield responsiveness of chicken hepatocytes to insulin at concentrations similar to those reported in live chickens.

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