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Molecular Effects of Nicarbazin on Avian Reproduction

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ABSTRACT Nicarbazin (NCZ) is an anticoccidial drug routinely used in the poultry industry that can negatively affect reproduction by reducing egg production, egg weight, and egg hatchability. The molecular mechanisms by which NCZ affects reproduction are unknown. Lipoprotein lipase, vitellogenin, transglutaminase, and calcium are all involved in egg formation and embryogenesis. Therefore, in vitro assays were used to evaluate 4 potential mechanisms of action of NCZ on egg formation and embryogenesis. First, a lipoprotein lipase assay was conducted to determine if NCZ increases lipoprotein lipase activity. Second, vitellogenin phosphorylation was evaluated to determine if NCZ acts as a vitellogenin phosphatase. Third, transglutaminase activity was measured to determine if NCZ inhibits transglutaminase activity. Finally, bull sperm was used as a model to determine if specific channel-mediated calcium uptake can be blocked by NCZ. Nicarbazin increased the activity of lipoprotein lipase in vitro at 3.9 and 7.8 μg of NCZ/mL. Nicarbazin increased intracellular calcium levels in bull sperm, suggesting it also acts as a calcium ionophore. The portion of the NCZ molecule responsible for the increase in intracellular calcium is 2-hydroxy-4,6-dimethylpyrimidine. Nicarbazin affected vitellogenin phosphorylation but only at a concentration many times higher than expected plasma values. Nicarbazin also inhibited transglutaminase activity in vitro. Whereas the 4,4′-dinitrocarbanilide portion of the NCZ molecule inhibited transglutaminase activity, the 2-hydroxy-4,6-dimethylpyrimidine portion increased transglutaminase activity. All of these assays were conducted in vitro; therefore these results should be viewed as preliminary findings to aid in directing further research on the effect of NCZ on reproduction in vivo. Because NCZ increases lipoprotein lipase activity and acts as a calcium ionophore, future experiments should investigate these effects in particular.

Key words: calcium ionophore, lipoprotein lipase, nicarbazin, transglutaminase, vitellogenin

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INTRODUCTION

Nicarbazin (**NCZ**) is an anticoccidial drug routinely used in the poultry industry since the 1950s to control protozoan cecal and intestinal infections by *Eimeria* species in broiler chickens. It is an equimolar complex consisting of 4,4′-dinitrocarbanilide (**DNC**) and 2-hydroxy-4,6-dimethylpyrimidine (**HDP**). The function of HDP is to increase absorption of the material in the gut, whereas DNC is the active anticoccidial drug (Cuckler et al., 1955; Rogers et al., 1983). When fed to laying hens, NCZ affects reproduction by reducing egg production, egg weight, and egg hatchability (Jones et al., 1990b; Hughes et al., 1991; Chapman, 1994).

Although the mechanisms by which NCZ reduces egg production and egg weight are unknown, NCZ may be preventing ova from maturing (Baker et al., 1957). Necropsy of hens fed a ration containing 90 ppm NCZ revealed that the largest follicle was absent from the ovary with no signs of recent ovulation or atresia (Baker et al., 1957). Luck (1979) also found ovaries without a follicular hierarchy and less well-developed oviducts in laying hens treated with 375 ppm NCZ in their feed. Although NCZ did not affect luteinizing hormone levels or pituitary responsiveness to luteinizing hormone releasing hormone, it decreased the sensitivity of the chicken hypothalamus to exogenous progesterone (Luck, 1979). Luck (1979) suggested that egg production is decreased because yolk deposition in the follicles is prevented. White Leghorn hens fed 400 to 700 ppm NCZ in feed exhibited reduced egg production concomitant with a 2-fold rise in plasma cholesterol concentrations, supporting Luck's hypothesis (Weiss, 1979).

Egg yolk is comprised of very low density lipoprotein (**VLDL**) and vitellogenin (**VTG**), both of which are produced in the liver in response to estrogen stimulation (Hillyard et al., 1956; Kudzma et al., 1979; Green, 1980; Shapiro, 1982; Wallace, 1985). The main constituent of egg yolk is VLDL. Although nonlaying hens and roosters have small amounts of serum VLDL, it is a major serum component in laying hens (Burley et al., 1984). One component of VLDL, apoVLDL-II, provides the VLDL parti-

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cles some resistance to degradation by lipoprotein lipase (**LL**; Griffin et al., 1982; Schneider et al., 1990). In addition, laying hen VLDL particles contain less apoC-II, a LL activator (Griffin et al., 1982; Griffin and Perry, 1985).

Vitellogenin is comprised of 1 lipovitellin and 2 phosvitin polypeptides (Deely et al., 1975; Chistmann et al., 1977). One of the posttranslational modifications VTG undergoes is the phosphorylation of serine residues on phosvitin (Wang and Williams, 1982). The phosphates confer a negative charge that allows phosvitin to bind calcium and iron (Allerton and Perlmann, 1965; Clark, 1970; Taborsky, 1980). Because of this, VTG is the main carrier of calcium and iron to the egg yolk (Morgan, 1975; Grunder et al., 1980; Lopez-Berjes et al., 1981). Dephosphorylation of these serine residues on phosvitin prevents the uptake of VTG into the follicle (Miller et al., 1982). Hens treated with 400 ppm NCZ in their feed exhibit reduced calcium binding by calcium binding protein and blood hypercalcemia (Bar and Hurwitz, 1971). This indicates that although VTG is produced, it is altered by NCZ somehow, preventing it from binding calcium.

Upon release into the blood stream, VLDL and VTG pass out of the capillaries surrounding the oocyte (Perry et al., 1978a,b) and through the granulosa cell layer of the oocyte. Once they reach the oolemma, they bind to the same 95 kDa receptor (George et al., 1987; Stifani et al., 1990; Barber et al., 1991). Clusters of occupied receptors induce the formation of clathin coated pits that are engulfed by the oolemma to become clathin coated vesicles (Wyburn et al., 1965; Schjeide et al., 1969). Transglutaminase assists in the formation of clathin coated pits, and inhibition of the enzyme prevents uptake of VTG (Tucciarone and Lanclos, 1981).

The molecular mechanism by which NCZ reduces egg hatchability is also unknown. However, NCZ may change the permeability of the vitelline membrane, creating an unfavorable environment for embryonic development (Polin, 1957; van Tienhoven et al., 1958; Cunningham, 1977). Laying hens fed NCZ produce eggs with mottled yolks (Baker et al., 1957: Polin et al., 1957, Jones et al., 1990a). Mottled yolks show a decrease in yolk solids (Cunningham, 1976), and exhibit an increase in albumen (Cunningham, 1977). In addition, yolk components such as fat, protein, calcium, phosphorus, and iron decrease in mottled yolks but increase in the albumen of eggs with mottled yolks (Cunningham, 1976: Cunningham, 1977).

We hypothesized that if NCZ increases LL activity, it could cause the degradation of VLDL in the blood prior to reaching the egg. Because VLDL is the major component of egg yolk, egg weight and egg production would decrease as a result. Phosphorylation of serine residues on VTG is necessary for calcium and iron binding and binding to the 95 kDa receptor. Therefore, we hypothesized that if NCZ acts as a phosphatase, it would prevent VTG from binding to the receptor, thus reducing egg weight and production. Additionally, there would be less calcium and iron available to the embryo, which could affect egg hatchability. We hypothesized that if NCZ inhibited transglutaminase (**TG**) activity, clathin coated pits could not form and uptake of yolk components would not occur, resulting in reduced egg weight and production. We also hypothesized that if NCZ acts as a calcium channel blocker, it could disrupt crucial ion gradients needed for proper egg formation and embryogenesis.

We chose to focus on the mechanisms described in the previous paragraph as potential targets of NCZ. The objective of this study was to determine the molecular mechanisms by which NCZ affects egg hatchability and egg production. We accomplished this by testing 4 hypotheses as follows: 1) NCZ increases LL activity; 2) NCZ acts as a VTG phosphatase; 3) NCZ inhibits TG activity; and 4) NCZ acts as a calcium channel blocker.

METHODS AND MATERIALS

Lipoprotein Lipase Assay

The LL assay was based on the principle that LL will cleave dibutyrlfluorescein (**DBF**), releasing fluorescein that can then be measured in a spectrofluorometer (Del Prado et al., 1994). Dibutyrlfluorescein was prepared as described previously by Del Prado et al. (1994). Briefly, 10 mL of pyridine (P4036, Sigma Chemical Co., St. Louis, MO), 30 mL of butyric anhydride (150540, Sigma Chemical Co.), and 10 mg of fluorescein (F6377, Sigma Chemical Co.) were mixed at 23°C for 8 min and then incubated in the dark for 24 h at room temperature. To this mixture was added 30 mL of 100% ethanol (111000200, Pharmco Products Inc., Brookfield, CT), and the mixture was incubated at –20°C for 23 h. The mixture was thawed at 23°C for 15 min, then mixed on a vortex mixer for 15 min to break up the crystals.

Solvent was removed using a vacuum flask and 5.5 cm, grade 362 filter paper (F2215-55, Baxter). The filtrate was washed with 95% ethanol until the solvent ran clear, and the DBF was stored in the dark at 4°C. A DBF stock solution was made by dissolving 10 mg of DBF in 50 mL of ethylene glycol monomethyl ether (**EGME**; E2632, Sigma Chemical Co.). A DBF working solution was made by mixing 5 mL of DBF stock solution with 100 mL of low potassium phosphate buffer (291 mOsm, pH = 7.09).

Disposable 12 × 75 mm borosilicate glass tubes (60825-913, VWR International, Aurora, CO) were used for the assay. To each test tube was added 1 mL of DBF working solution and 15 μg of LL (1 μg LL/μL of Dulbecco's PBS). In 2 separate tubes, 10 μL of LL inhibitors, AA861 (0.03 *M*; A3711, Sigma Chemical Co.), or nordihydroguaiaretic acid (0.1 *M*; N5023, Sigma Chemical Co.) in EGME were added as negative controls. To 1 of 4 other tubes was added 10 μL of 1, 2, 4, or 8 μg of NCZ (Phibro Animal Health Inc., Fairfield, NJ)/10 μL of dimethyl sulfoxide (**DMSO**; D5879, Sigma Chemical Co.). Two tubes containing 10 μL of DMSO or EGME served as controls for the NCZ or inhibitor tubes, respectively. A test tube with only DBF and LL served as a positive control. A test tube with DBF and no LL served as a blank to monitor background fluorescence. The solution in each test tube

was mixed briefly on a vortex mixer, then incubated in a water bath at 37°C.

Tubes were removed after 1 min of incubation, and the amount of fluorescein released was determined by measuring fluorescence with a Turner model 450 spectrofluorometer. The spectrofluorometer was zeroed first using a blank tube consisting of DBF working solution only, and the gain was set to 1. The excitation wavelength was set at 490 nm, and the emission wavelength was set at 535 nm. After obtaining readings, the test tubes were returned to the water bath. Tubes were removed for subsequent readings at 2 min intervals until 11 min of incubation time had passed. The experiment was replicated 5 times.

Vitellogenin Phosphorylation Assay

Phosphorylation of VTG was assessed using a purchased phosphoprotein stain (Molecular Probes Inc., Eugene, OR). Plasma samples were obtained by drawing 3 mL of blood from the brachial vein of laying and nonlaying chickens. Plasma samples were pooled to standardize the amount of VTG in each sample. The plasma of nonlaying chickens was used as a negative control. Positive controls consisted of laying hen plasma only or laying hen plasma plus 10 μL of DMSO. Just prior to starting the assay, fresh NCZ, DNC (390151, Aldrich Chemical Co., Milwaukee, WI), and HDP (22588-6, Aldrich Chemical Co.) solutions were made. To 100 μL of laying hen plasma was added 10 μL of 1, 2, 4, or 8 μg of NCZ/10 μL of DMSO; 1, 2, 4, or 8 μg of DNC/10 μL of DMSO; or 1, 2, 4, or 8 μg of HDP/10 μL of water. Samples were mixed and incubated at 4°C for 30 min. After incubation, all plasma samples were diluted 1:100 in PBS (P4417, Sigma Chemical Co.). A standards solution was prepared by mixing 2 μL of PeppermintStick standard (P33350, Molecular Probes Inc.) with 38 μL of ultra pure water.

A 1× SDS-Tris-glycine running buffer was made by adding 70 mL of 10× SDS-glycine (161-0732, BioRad Laboratories, Hercules, CA) to 630 mL of ultra pure water. Fixing solution consisted of 100 mL of methanol (A433P-4, Fisher Scientific, Fair Lawn, NJ), 20 mL of acetic acid (45726, Sigma-Aldrich, St. Louis, MO), and 80 mL of deionized water. A ProQ Diamond destaining solution was made by mixing 187.5 mL of deionized water, 50 mL of acetonitrile (494445, Sigma-Aldrich), and 12.5 mL of 1 *M* sodium acetate (110191, Aldrich Chemical Co.).

Sample buffer (3×, 20 μL; 87703S, New England BioLabs Inc., Ipswich, MA) was added to 40 μL of plasma dilutions and to the standards solution. Samples were mixed briefly and centrifuged for 5 s at 8 to 10 × G. Samples were heated for 5 min at 95°C, then centrifuged again for 5 s at 8 to 10 × G. A 4 to 20% Tris-glycine-SDS minigel (81002-006, VWR International, Aurora, CO) was covered with 1× SDS-Tris-glycine running buffer. Each well was loaded with 50 μL of sample, and the plasma proteins were separated by gel electrophoresis at 150 V for 90 min.

The gel was removed from the electrophoresis apparatus, covered with 100 mL of fixing solution, and incubated by gently agitating at 23°C for 30 min. The gel was washed twice by covering it with 100 mL of ultra pure water and gently agitating at 23°C for 10 min. The gel was then covered with 50 mL of ProQ Diamond phosphoprotein stain (P33300, Molecular Probes Inc.) and incubated in the dark with gentle agitation at 23°C for 2 h. The phosphoprotein stain was removed, and 80 mL of ProQ Diamond destaining solution was added to the gel. The gel was incubated in the dark with gentle agitation at 23°C for 1 h. The destaining step was repeated once.

Images of the gel were produced on an Epichemi3 Darkroom 2UV benchtop transilluminator (UVP Bioimaging Systems, Ultraviolet Products Ltd., Cambridge, UK) using an ethidium bromide filter (excitation = 365 nm, emission = 570 to 640 nm). Digital images were analyzed by densitometry using Scion Image for Windows (Scion Corporation, Frederick, MD). The experiment was replicated 5 times.

A Western blot was used to confirm the presence and position of VTG on the gel. Briefly, plasma samples from a laying hen and from a male were diluted 1:100 in PBS and applied to a 4 to 20% Tris-glycine-SDS minigel with sample buffer. Proteins were separated for 90 min at 150 V using SDS-PAGE. Proteins were transferred to a nitrocellulose membrane in transfer buffer for 60 min at 100V. The membrane was blocked for 30 min at 23°C with gentle agitation using blocking buffer consisting of Tris buffered saline (**TBS**) and 5% milk powder. The membrane was then incubated with 1:1000 rabbit anti-VTG antibody (D. Williams, Pharmacological Sciences, SUNY, Stony Brook, NY) in blocking solution for 2 h at 23°C with gentle agitation. The membrane was washed once with TBS containing 0.05% Tween 20 (vol/vol; P1379, Sigma Chemical Co.), and twice with TBS.

The membrane was then incubated with alkaline phosphatase labeled goat anti-rabbit IgG antibody (1:1,000; A7778, Sigma Chemical Co.) in blocking buffer for 60 min at 23°C with gentle agitation. The membrane was washed once with TBS-Tween 20 and twice with TBS. Color was developed by incubating the membrane in alkaline phosphatase substrate (pH 9.5; B5655, Sigma Chemical Co.) containing 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate/mL, 0.3 mg of Nitro blue tetrazolium/mL, 100 m*M* Tris buffer, and 5 m*M* magnesium chloride. The reaction was stopped after 10 min by washing the membrane in deionized water.

TG Assay

The TG activity was assessed using an assay previously described by Lilley et al. (1997). The assay measures the protein crosslinking activity of TG based upon incorporation of biotin-labeled casein into unlabeled casein that is bound to microtiter plates.

Casein was biotinylated using a procedure previously described for labeling antibodies with biotin (Harlow and Lane, 1988). A 0.1 *M* sodium borate buffer was prepared by dissolving 7 g of boric acid (B6768, Sigma Chemical Co.) and 10 g of sodium tetraborate (B0127, Sigma Chemi-

cal Co.) in 1 L of deionized water and titrating the solution to pH 8.8. A solution of 3 mg of N′,N′-dimethylcasein/mL (C9801, Sigma Chemical Co.) was prepared in 0.1 *M* sodium borate buffer. A solution of 3 mg of N-hydroxysuccinimide biotin/mL (H1759, Sigma Chemical Co.) was prepared in DMSO.

The casein and biotin solutions were combined in a 9:1 casein:biotin ratio and incubated at 23°C for 4 h. Ammonium chloride (1 *M*; A4515, Sigma Chemical Co.) was added to the biotin ester solution at a rate of 20 μL per 250 μg of biotin ester. The solution was incubated for 10 min at 23°C, then dialyzed against PBS overnight in #3 Spectra/Por dialysis tubing (132724, Spectrum Medical Industries, Los Angeles, CA). The biotinylated casein was stored at –70°C until use.

Flat bottom 96 well microtiter plates (3455, Thermo LabSystems, Franklin, MA) were coated with 50 ng/well N,N′dimethylcasein in 50 m*M* sodium carbonate buffer (C3041, Sigma Chemical Co.) at pH 9.8 (100 μL/well) and incubated at 37°C for 1 h. Plates were washed twice with PBS containing 0.05% Tween 80 (vol/vol; P8074, Sigma Chemical Co.), and twice with deionized water.

Plates were blocked with 300 μL/well BSA (1 mg/mL) in 50 m*M* sodium carbonate buffer for 30 min at 23°C. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 100 m*M* Tris-HCl (pH 8.5; T3253, Sigma Chemical Co.). Each plate was then incubated overnight at 37°C with 100 μL/well 100 m*M* Tris-HCl containing 5 m*M* calcium chloride (C4901, Sigma Chemical Co.), 10 m*M* dithiotheitol (D0632, Sigma-Aldrich), 37.5 m*M* putrescine (D13208, Aldrich Chemical Co.), and 0.25% TG (wt/vol; T5398, Sigma Chemical Co.). Plates were removed from the incubator and washed twice with PBS-Tween 80, twice with deionized water, and once with 100 m*M* Tris-HCl.

To each plate was added 100 μL/well 100 m*M* Tris-HCl containing 5 m*M* calcium chloride, 10 m*M* dithiotheitol, 0.75 μg/mL biotinylated casein, and 0.5% TG (wt/vol). In addition, 10 μL/well of Tris-HCl, DMSO, 1:200 goat anti-TG antibody (T7066, Sigma Chemical Co.), NCZ, DNC, or HDP were added to the appropriate wells. The NCZ and DNC solutions consisted of 1, 2, 4, or 8 μg of NCZ or DNC/10 μL of DMSO, and the HDP solutions consisted of 1, 2, 4, or 8 μg of HDP/10 μL of water. All NCZ, DNC, and HDP solutions were made just prior to starting the assay. Each plate had 6 wells per treatment group. Wells containing only Tris-HCl were used as negative controls. Plates were incubated for 1 h at 37°C. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 100 m*M* Tris-HCl.

A 1:625 dilution of extravidin peroxidase (E2886, Sigma Chemical Co.) was added to each well (100 μL/well), and plates were incubated at 37°C for 45 min. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 0.05 *M* phosphate-citrate buffer (pH 5.0; P9305, Sigma Chemical Co.) containing 0.014% hydrogen peroxide.

A 3,3′,5,5′-tetramethylbenzidine (T3405, Sigma Chemical Co.) solution was made by dissolving 1 3,3′,5,5′-tetramethylbenzidine tablet per 10 mL of 0.05 *M* phosphate-citrate buffer. The 3,3′,5,5′-tetramethylbenzidine solution was added to each well (100 μL/well), and color was allowed to develop. The reaction was terminated after 2 to 3 min using 100 μL/well 2 *M* sulfuric acid. Plates were read at 450 nm on an Ultramark Microplate Imaging System (170-9500, BioRad Laboratories). The experiment was replicated 5 times, with 1 plate per replication.

Calcium Channel Assay

Bull sperm were used as a model for this experiment because sperm contain L-type calcium channels similar to those found in avian follicular cells (Schwartz et al., 1989; Goodwin et al., 2000). In addition, millions of sperm can be obtained without extensive purification, which can alter a cell's membrane function. A large influx of intracellular calcium though calcium channels occurs in sperm during capacitation, and this influx can be induced in vitro by incubating sperm with progesterone (Kobiri et al., 2000). The influx of intracellular calcium can be monitored using flow cytometry.

Bull tyrodes solution was made by dissolving 5.69 g of sodium chloride (S7653, Sigma Chemical Co.), 0.23 g of potassium chloride (P3911, Sigma Chemical Co.), 0.04 g of sodium phosphate (S0876, Sigma Chemical Co.), 2.09 g of sodium bicarbonate (S5761, Sigma Chemical Co.), 0.29 g of calcium chloride dihydrate (C5080, Sigma Chemical Co.), and 0.08 g of magnesium chloride hexahydrate (M2670, Sigma Chemical Co.) in nanopure water. Bull Tyrode's albumin-lactate-pyruvate diluent was made by dissolving 0.0022 g of sodium pyruvate (P2256, Sigma Chemical Co.), 0.368 mL of sodium lactate (L1375, Sigma Chemical Co.), 0.09 g of glucose (G7528, Sigma Chemical Co.), 0.238 g of N-(2-hydroxyethyl)piperazine-N′2-ethanesulfonic acid (H3375, Sigma Chemical Co.), and 0.3 g of BSA (A2153, Sigma Chemical Co.) in 100 mL of bull tyrodes solution.

Bull sperm were diluted to 50×10^6 cells/mL in bull Tyrode's albumin-lactate-pyruvate diluent, and 2 mL of the diluted sperm was added to each sample tube. Sperm in all sample tubes except the control tubes were stained with 10 μ*M* Fluo-3 AM (an intracellular calcium indicator; F1241, Invitrogen, Carlsbad, CA) and 5 μ*M* propidium iodide (a stain to detect dead cells; P1304MP, Invitrogen). There were 3 control tubes consisting of Fluo-3 AM stain only, propidium iodide stain only, and both stains.

To each sample tube, 20 μL of DMSO, 80 μ*M* nifedipine (calcium channel inhibitor; N7634, Sigma Chemical Co.), 4.75 μ*M* A23187 (calcium ionophore; C5149, Sigma-Aldrich), NCZ, DNC, or HDP solutions were added. The NCZ solutions consisted of 1, 2, 4, or 8 μg of NCZ/20 μL of DMSO. The DNC solution consisted of 8 μg of DNC/20 μL of DMSO. The HDP solution consisted of 8 μg of HDP/20 μL of water.

Tubes were mixed briefly using a vortex mixer, then incubated for 20 min at 23°C in the dark. A 0.5-mL subsample was analyzed on an Epics V flow cytometer (Coulter Electronics, Miami, FL) with the argon laser

tuned to 488 nm to excite Fluo-3 AM and propidium iodide. The filter setup included a 457 to 505-nm laser blocker, a 550-nm dichoic beam splitter, a 525 to 560-nm band-pass filter to detect Fluo-3 AM, and a 610-nm long-pass filter to detect propidium iodide. To the remainder of the samples was added 800 μL of 40 μ*M* progesterone in DMSO. The samples were mixed briefly on a vortex mixer and then were incubated at 37°C for 1 h. Subsamples (0.5 mL) were taken every 15 min during the hour of incubation for analysis on the flow cytometer. This experiment was replicated 5 times.

Statistical Analysis

Lipoprotein Lipase Assay. The absorbance value for the blank test tube was subtracted from the absorbance for all other tubes in the same time period. The difference between the absorbance of the LL positive control and the DMSO control was subtracted from all test tubes containing DMSO in the same time period. The difference between the absorbance of the LL positive control and the EGME control was subtracted from all test tubes containing EGME in the same time period. The adjusted absorbances were used to standardize the data by calculating a percent of the LL positive control. Absorbances for each tube were divided by the absorbance for the LL positive control in the same time period, and the result was multiplied by 100 to obtain a percentage of the positive control. The standardized percentages were analyzed as a mixed effects model (PROC MIXED, SAS Institute Inc., Cary, NC), and significance was defined as $P \leq 0.05$. Means separations were carried out using PDMIX800 (Saxton, 1998).

Vitellogenin Phosphorylation Assay. To obtain a mean background value for each gel, the mean optical density of the area of the gel corresponding to the VTG band was averaged for the nonlaying chicken plasma and PeppermintStick standard lanes. The mean background value was subtracted from the mean optical density of the VTG bands for each gel to create an adjusted density. The adjusted density for each VTG band was compared with the adjusted density of the VTG band for laying chicken plasma on the same gel to obtain a percentage of the control. The percentages of the control were analyzed by ANOVA (PROC GLM, SAS Institute), and significance was defined as $P \leq 0.05$. Means were separated using the least significant difference.

Transglutaminase Assay. The absorbances of the blank wells were averaged, and the mean absorbance was subtracted from the absorbance of each well to eliminate background fluorescence. For each plate, all 6 wells in each treatment group were averaged. The average absorbance for each treatment group was divided by the average absorbance for the DMSO control group for that plate. The result was multiplied by 100 to obtain a percentage of the DMSO control. The percentages of the DMSO control were used for analysis by ANOVA (PROC GLM, SAS Institute), and significance was defined as $P \leq 0.05$. Means were separated using the least significant difference.

Table 1. Effect across time of addition of 10 μL of 1, 2, 4, or 8 μg/10 μL of nicarbazin (NCZ) in dimethyl sulfoxide (DMSO), lipoprotein lipase (LL) inhibitors AA861 (0.03 *M*), and nordihydroguaiaretic acid (NDGA; 0.1 *M*) in ethylene glycol monomethyl ether (EGME), or DMSO to test tubes containing 1 mL of dibutyrlfluorescein (DBF) and 15 μg of LL on LL activity in vitro after 1, 3, 5, 7, 9, and 11 min of incubation at 37°C

Treatment	n	Mean percentage of DMSO control
DMSO control	30	100.0[d]
0.03 *M* AA861	30	–17.7[e]
0.1 *M* NDGA	30	10.9[d]
1 μg of NCZ	30	107.3[c]
2 μg of NCZ	30	108.6[c]
4 μg of NCZ	30	169.4[b]
8 μg of NCZ	30	233.4[a]
SEM	8.4	

[a–e]Means within the column with different subscripts are significantly different ($P \leq 0.05$).

Calcium Channel Assay. Data were standardized by calculating a percentage of the DMSO control for the percentage of cells with low intracellular calcium, the percentage of cells with high intracellular calcium, and the percentage of dead cells. The percentage of cells with low intracellular calcium in each group was divided by the percentage of cells with low intracellular calcium in the DMSO control group for the same time period. The result was multiplied by 100 to obtain a percentage of the DMSO control. The same procedure was used to calculate a percentage of the DMSO control for the percentage of cells with high intracellular calcium and the percentage of dead cells. The standardized percentages were analyzed as a mixed effects model (PROC MIXED, SAS Institute), and significance was defined as $P \leq 0.05$. Means separations were carried out using PDMIX800 (Saxton, 1998).

RESULTS

Nicarbazin significantly increased LL activity (Table 1). However, LL activity decreased over time ($P \leq 0.05$); most of the change occurred during the first 3 min of incubation. A significant treatment × period interaction also existed ($P \leq 0.05$). Changes in LL activity during the remainder of the incubation period were slight. Both AA861 and NDGA inhibited LL activity, giving 100 and 89% inhibition, respectively.

Vitellogenin phosphorylation differed among treatments (Table 2). Whereas DMSO decreased the amount of VTG phosphorylation by 19.5% compared with the control, NCZ and DNC were not significantly different from the control or from DMSO. Although treatment with 1, 2, and 4 μg of HDP decreased the amount of VTG phosphorylation compared with the control, they were not different from DMSO.

Transglutaminase activity also differed among treatments (Table 3). Whereas HDP tended to increase the activity of TG by 30 to 40%, NCZ and DNC tended to

Table 2. Effect of addition of 10 μL of 1, 2, 4, or 8 μg/10 μL of nicarbazin (NCZ) in dimethyl sulfoxide (DMSO), 1, 2, 4, or 8 μg/10 μL of 4,4′-dinitrocarbanilide (DNC) in DMSO, 1, 2, 4, or 8 μg/10 μL of 4,6-dimethylpyrimidine (HDP) in water, or DMSO to 100 μL of chicken plasma on phosphorylation of vitellogenin[1]

Treatment	n	Mean percentage of laying hen control
Laying control	12	100.0[a]
DMSO	5	80.5[bcd]
1 μg of NCZ	5	93.6[ab]
2 μg of NCZ	5	86.3[abcd]
4 μg of NCZ	5	92.6[abc]
8 μg of NCZ	5	89.3[abc]
1 μg of DNC	5	83.3[bcd]
2 μg of DNC	5	88.0[abcd]
4 μg of DNC	5	90.6[abc]
8 μg of DNC	5	92.9[abc]
1 μg of HDP	5	79.9[bcd]
2 μg of HDP	5	79.2[cd]
4 μg of HDP	5	75.3[d]
8 μg of HDP	5	93.1[abc]
SEM	4.9	

[a–d]Means within the column with different subscripts are significantly different ($P \leq 0.05$).

[1]Plasma was diluted 1:100 in PBS, and proteins were separated on a 4 to 20% Tris-glycine minigel for 90 min at 150 V. Phosphoproteins were stained with ProQ Diamond phosphoprotein stain (33300, Molecular Probes, Eugene. OR).

decrease the activity of TG by 30 to 61.5%. The anti-TG antibody inhibited TG activity by 66%.

As shown in Table 4, there was a significant treatment effect on the percentages of cells having high intracellular calcium but not on the percentages of cells having low intracellular calcium ($P = 0.4737$). The percentage of cells having low intracellular calcium tended to increase over time ($P \leq 0.05$), whereas the percentage of cells having high intracellular calcium tended to decrease over time ($P \leq 0.05$). There was a significant treatment × time interaction effect for the percentage of cells having high intracellular calcium ($P \leq 0.05$; Table 4). The effects of NCZ on intracellular calcium levels occurred within the first 15 min of incubation. Treatment significantly affected the percentage of dead cells ($P \leq 0.05$), with A23187 and nifedipine inducing the highest percentages of dead cells. The percentages of dead cells in the NCZ, DNC, and HDP groups were not different from the controls with and without DMSO. As expected, the percentage of dead cells increased over time ($P \leq 0.05$)

Table 3. Effect of addition of 10 μL of 1, 2, 4, or 8 μg/10 μL of nicarbazin (NCZ) in dimethyl sulfoxide (DMSO), 4,4′-dinitrocarbanilide (DNC) in DMSO, 4,6-dimethylpyrimidine 'HDP) in water, 1:200 goat antitransglutaminase antibody in PBS, or DMSO to microtiter plates containing 100 μL/well Tris-HCl solution consisting of 5 m*M* calcium chloride, 10 m*M* dithiotheitol, 0.75 μg/mL of biotinylated casein, and 0.5% transglutaminase (w:v) on transglutaminase (TG) activity in vitro

Treatment	n	Mean percent of DMSO control
DMSO control	5	100.0[b]
Anti-TG antibody	5	33.6[e]
1 μg of NCZ	5	70.2[c]
2 μg of NCZ	5	55.6[cd]
4 μg of NCZ	5	38.5[de]
8 μg of NCZ	5	43.2[de]
1 μg of DNC	5	65.0[d]
2 μg of DNC	5	55.1[cd]
4 μg of DNC	5	51.0[cde]
8 μg of DNC	5	51.9[cde]
1 μg of HDP	5	141.1[a]
2 μg of HDP	5	131.2[a]
4 μg of HDP	5	132.4[a]
8 μg of HDP	5	135.2[a]
SEM	7.2	

[a–e]Means within the column with different subscripts are significantly different ($P \leq 0.05$).

Table 4. Effect of addition of 20 μL of 1, 2, 4, or 8 μg/20 μL of nicarbazin (NCZ) in dimethyl sulfoxide (DMSO), 8 μg/20 μL of 4,4′-dinitrocarbanilide (DNC) in DMSO, 8 μg/20 μL of 4,6-dimethylpyrimidine (HDP) in water, 80 μ*M* nifedipine (calcium channel inhibitor), 4.75 μ*M* A23187 (calcium ionophore), or DMSO to test tubes containing 2 mL of bull sperm in Tyrode's albumin-lactate-pyruvate diluent (50 × 106 cells/mL) stained with 10 μ*M* Fluo-3 AM and 5 μ*M* propidium iodide on the percentage of sperm cells having high intracellular calcium 15 min after the addition of 800 μL of 40 μ*M* progesterone and incubation at 37°C

	0 min		15 min	
Treatment	n	Mean[1]	n	Mean
DMSO control	5	100.0[defghijk]	5	100.0[defghijk]
Nifedipine	5	97.8[defghijk]	5	60.0[jk]
A23187	5	237.5[b]	5	297.5[a]
1 μg of NCZ	5	143.3[cdf]	5	113.1[cdefghij]
2 μg of NCZ	5	142.7[cdef]	5	127.2[cdefghi]
4 μg of NCZ	5	134.9[cde]	5	84.3[fghijkl]
8 μg of NCZ	5	131.9[cdefg]	5	112.8[cdefghij]
8 μg of DNC	4	114.0[cdefghij]	4	86.1[defghijk]
8 μg of HDP	4	167.5[c]	4	114.1[cdefghij]
SEM	22.5			

[a–j]Means within columns with the different subscripts are significantly different ($P \leq 0.05$).

[1]Means are percentage of the DMSO control.

DISCUSSION

Nicarbazin increased the activity of LL in vitro in the 4 and 8 μg treatment groups. The total assay volume in each test tube was 1.025 mL, giving a concentration of 3.9 μg/mL and 7.8 μg/mL in the 4 and 8 μg of NCZ treatment groups, respectively. The entire NCZ molecule has a molecular weight of 426.38, whereas the DNC portion has a molecular weight of 292.25 (Wells, 1999), which is 68.5% of the NCZ molecule. Therefore, 4 μg of NCZ contains 2.74 μg of DNC and 8 μg of NCZ contains 5.48 μg of DNC. The concentration of DNC in the assay was therefore 2.67 and 5.35 μg/mL in the 4 and 8 μg of NCZ treatment groups, respectively. These values are within the range expected in the plasma of waterfowl fed NCZ-treated bait at 31 to 49 mg of NCZ/kg of BW. A study of mallards fed at these dose levels showed peak plasma DNC levels were 2.7 to 5.4 μg/mL (Yoder et al., 2006a).

Chickens fed 400 mg of NCZ/kg of feed had peak plasma DNC levels of approximately 3 μg/mL and a 71% reduction in egg production (Ott et al., 1956). Several studies found feeding 125 mg of NCZ/kg of feed reduced egg production significantly (Baker et al., 1957; McLoughlin et al., 1957; Jones et al., 1990c). A comparative gavage study showed treatment of chickens with NCZ at 125

ppm produced a peak plasma DNC level of 2.9 μg/mL (Yoder et al., 2005). These plasma DNC levels are comparable to the concentrations used in the in vitro assay.

The increased activity of LL due to NCZ could cause premature degradation of VLDL while in the blood, resulting in a decrease of lipid being deposited into the yolk, thereby decreasing overall egg weight and production. Baker et al. (1957) suggested NCZ might prevent ova from maturing. Necropsy of hens fed a ration containing 90 ppm NCZ revealed that the largest follicle was absent with no signs of recent ovulation or atresia (Baker et al., 1957). Luck (1979) also found ovaries without a follicular hierarchy and less well-developed oviducts in laying hens treated with 375 ppm NCZ in feed.

Nicarbazin did not affect luteinizing hormone levels or pituitary responsiveness to luteinizing hormone releasing hormone but decreased the sensitivity of the chicken hypothalamus to exogenous progesterone (Luck, 1979). Luck (1979) suggested that egg production is decreased because yolk deposition in the follicles is prevented. White Leghorns fed 400 to 700 ppm NCZ in feed exhibited reduced egg production concomitant with a 2-fold rise in plasma cholesterol concentrations, supporting Luck's hypothesis (Weiss, 1979). These studies support our hypothesis that the increased LL activity due to NCZ causes premature degradation of VLDL. Future studies should investigate the effect of NCZ treatment on the activity of LL in vivo.

Although a statistically significant effect of NCZ on phosphorylation of VTG was found, this effect is probably not biologically significant. The total assay volume of plasma plus treatment was 0.11 mL. If DNC comprises 68.5% of the NCZ molecule, then HDP must comprise 31.5% of the NCZ molecule. Using these figures, the concentrations of DNC and HDP in this assay ranged from 9.1 to 72.7 μg/mL in the DNC and HDP groups. The concentration of DNC in the NCZ groups ranged from 6.2 to 49.8 μg/mL, and the concentration of HDP in the NCZ groups ranged from 2.8 to 22.9 μg/mL. This is many times higher than what would be expected in plasma. A decrease in VTG phosphorylation was caused by DMSO by itself. Although the NCZ and DNC groups had VTG with a greater degree of phosphorylation than the DMSO group, they were not significantly different from the DMSO group. The HDP group appeared to have no effect except at the 8-μg level.

There was a very large amount of VTG on the gels, which might make it difficult to detect small changes in phosphorylation. The assay could be rerun with plasma diluted at least 1:1,000 in PBS. However, such small changes would not likely be biologically significant. A more appropriate experiment would be to treat laying hens with NCZ and compare the phosphorylation of VTG from the plasma of treated and control hens.

Nicarbazin did have an inhibitory effect on TG in vitro. The portion of the NCZ molecule that appears to be responsible for this effect is DNC. Both DNC and NCZ decreased TG activity compared with the DMSO control, whereas HDP increased TG activity.

The total assay volume used in each well was 0.11 mL. Again, the concentrations of DNC and HDP ranged from 9.1 to 72.7 μg/mL in the DNC and HDP groups, much higher than what would be expected in plasma. The concentration of DNC in the NCZ groups ranged from 6.2 to 49.8 μg/mL, and the concentration of HDP in the NCZ groups ranged from 2.8 to 22.9 μg/mL. The same inhibitory effect might not occur at lower levels. Only the 4 and 8 μg of NCZ and DNC groups produced a decrease in TG activity similar to the anti-TG antibody. We chose to use the higher concentrations of NCZ, DNC, and HDP for this experiment because of the difficulty of accurately measuring such small quantities of NCZ, DNC, and HDP.

The total assay volume used in the calcium assays was 2.82 mL. The concentrations of DNC used ranged from 0.2 to 1.9 μg/mL in the NCZ groups and was 2.8 μg/mL in the DNC group. The concentrations of DNC in the DNC and 8 μg of NCZ groups are comparable to what is expected in plasma, whereas the concentrations in the remaining NCZ groups are lower than expected plasma values. The concentrations of HDP ranged from 0.1 to 0.9 μg/mL in the NCZ groups and 2.8 μg/mL in the HDP group. The concentration of HDP in the 8 μg of NCZ group is close to expected plasma values, whereas the concentrations in the remaining NCZ groups are lower than expected. The concentration in the HDP group is higher than expected. Wells (1999) reported a range of HDP concentrations from 1.07 to 2.07 μg/mL in chickens fed 125 ppm NCZ for 7 d.

The effects of NCZ on intracellular calcium levels occurred within the first 15 min of incubation. No significant effects on intracellular calcium levels were observed after 30 min of incubation. The HDP group consistently had a greater percentage of sperm cells with high intracellular calcium than the DMSO control, indicating it is acting as an ionophore. However, as has already been pointed out, the concentration of HDP used in that group is slightly higher than expected plasma values. Fifteen minutes after the addition of progesterone, the NCZ groups had a higher percentage of cells with high intracellular calcium than the DMSO control. The percentage of cells with high intracellular calcium was comparable in the NCZ and HDP groups. Because the concentrations of HDP in the NCZ groups were lower than expected plasma values, it seems reasonable to conclude that NCZ acts as an ionophore. The portion of the NCZ molecule responsible for ionophore activity is HDP. As compared with the DMSO control, the DNC group had comparatively fewer sperm cells with high intracellular calcium, indicating it may act as a weak calcium channel blocker.

The apparent activity of NCZ as an ionophore may help explain damage to the vitelline membrane in NCZ-treated hens that leads to egg yolk mottling and a reduction in egg hatchability. As an ionophore, NCZ could insert itself into the vitelline membrane, making the membrane more permeable. Evidence that vitelline membranes from NCZ-treated hens are more permeable was shown by Cunningham (1976, 1977). Cunningham found that mottled yolks from NCZ-treated hens exhibited a

decrease in yolk solids (1976) and an increase in egg albumen (1977). The percentages of fat, protein, ash, calcium, phosphorus, and iron are also reduced in mottled yolks (Cunningham, 1976), but these components increased in the egg albumen (Cunningham, 1977). Mottled yolks also contain the egg white proteins ovalbumin and conalbumin (Cunningham, 1976). In addition, vitelline membranes from NCZ-treated hens show degeneration at the microscopic level (Yoder et al., 2006b).

Although these assays examined the effects of NCZ in vitro, they provide some clues as to the mechanism by which NCZ affects reproduction. One of the main effects of NCZ on reproduction is to increase the activity of LL, thereby decreasing the amount of VLDL deposited into the follicle. The other main effect is the activity of NCZ as an ionophore to increase the permeability of the vitelline membrane. These assays should be viewed as preliminary studies to aid in directing further research on the effect of NCZ on reproduction in vivo.

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