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Role of AMPK in the Upregulation of Steroidogenic Acute Regulatory Protein in the Zona Fasciculata of the Adrenal Cortex

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Role of AMPK in the Upregulation of Steroidogenic Acute Regulatory Protein in the Zona Fasciculata of the Adrenal Cortex

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

Role of AMPK in the Upregulation of Steroidogenic Acute

Regulatory Protein in the Zona Fasciculata

of the Adrenal Cortex

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Physiology and Developmental Biology

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Cortisol is a glucocorticoid produced by the zona fasciculata (ZF) of the adrenal cortex. Traditionally, cortisol production and release was seen as being regulated strictly by adrenocorticotropic hormone (ACTH). While this is true of baseline cortisol levels and in response to acute mental stress, the picture is somewhat more complicated in other situations.

Interleukin-6 (IL-6) contributes to the maintenance of cortisol levels in situations of prolonged immune or inflammatory stress. AMP activated protein kinase (AMPK) was investigated as a possible mediator of the action of IL-6 or as an independent actor in raising cortisol levels in response to hypoxemic or hypoglycemic stress.

5-aminoimidazole-4-carboxamide 1-b-D-ribofuranoside (AICAR) was used to activate AMPK. Bovine ZF tissue fragments were exposed to AICAR alone and together with a known AMPK inhibitor, compound C. Protein or mRNA was then extracted from these tissue fragments. As an indicator of overall steroidogenic activity, these extracts were tested using RT-PCR and western blot assays for relative protein and mRNA levels of steroidogenic acute regulatory (StAR) protein, steroidogenic factor-1 (SF-1), and dosage sensitive sex reversal adrenal hypoplasia congenita gene on the X chromosome, gene 1 (DAX-1). Also a reporter gene assay was performed on H295R cells with a transfected StAR promoter.

In bovine ZF tissue fragments, AICAR caused a significant increase of StAR protein and mRNA and SF-1 protein with a decrease of DAX-1 protein in a dose and time dependant manner. DAX-1 mRNA was shown to decrease in response to AICAR administration in a dose dependant manner. AICAR induced increases in StAR protein and SF-1 protein, and the

attendant decrease in DAX-1 protein were all shown to be reduced by administration of compound C. This demonstrated that in this situation AICAR is acting through AMPK. When IL-6 was given with compound C the levels of StAR, SF-1, and DAX-1 were significantly reduced from samples treated with IL-6 alone. AICAR exposure also increased StAR promoter activity in a dose and time dependant manner. This AMPK induced increase in steroidogenic activity provides a possible mechanism for increased cortisol during hypoxia and hypoglycemia, and a possible mediator for IL-6 in the ZF.

Keywords: ACTH, Adrenal, Cortex, AMPK, Zona Fasciculata, ZF, Cortisol, StAR, Hypoxia, Hypoglycemia, SF-1, DAX-1, IL-6, Steroidogenesis

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TABLE OF CONTENTS

INTRODUCTION

Cortisol is released from the adrenal cortex and plays a key role in mediating the physiological response to stress. Various pathophysiological conditions result if cortisol release is either elevated (i.e. inhibition of immune function, emotional instability, coronary artery disease) or inhibited (i.e. autoimmune and inflammatory diseases, low blood glucose, collapse of the circulatory system resulting in shock). The release of cortisol during acute stress is regulated by the hormone ACTH from the anterior pituitary. However, during chronic stress related to inflammation and infection, ACTH's role in regulating cortisol release has been demonstrated to be diminished because plasma cortisol concentrations stay elevated whereas ACTH concentrations are normal (1). During chronic inflammatory stress the release of cortisol appears to be mediated by various cytokines (protein hormones that are released from several tissues of the body including the immune system and adipose tissue) (2). Particularly, the cytokine interleukin-6 (IL-6) has been shown to play a key role in regulating the release of cortisol during chronic stress related to inflammation and infection (3). We have found that this IL-6 induced increase in cortisol secretion is mediated in part through IL-6 upregulating the expression of the steroidogenic acute regulatory protein (StAR). The action of StAR is the rate limiting step in the synthesis of most steroids including cortisol (4). Control of transcription of StAR and many other steroidogenic proteins has been closely influenced by steroidogenic factor-1 (SF-1) and dosage sensitive sex reversal adrenal hypoplasia congenita gene on the X chromosome, gene 1 (DAX-1). While SF-1 up-regulates steroidogenic transcription, including StAR, DAX-1 has an opposing effect (5).

Cortisol helps maintain the body's responsiveness to stress, and one important role in this buildup of stress responsiveness is the maintenance of blood glucose. Cortisol accomplishes this

partially by reducing insulin sensitivity and increasing sensitivity to epinephrine. ACTH is elevated during hypoglycemia (6, 7), and this in turn would stimulate the zona fasciculata and would cause increased cortisol in order to try and bring blood sugar back up. There is some evidence that cortisol increase in response to hypoglycemia can be attributed to more than ACTH alone (8). It has been found that AMP activated protein kinase (AMPK) can act as the sensor of blood sugar levels in the hypothalamus that eventually leads to greater ACTH release (9). We have recently found that AMPK is present in the adrenal cortex, and we wanted to investigate if AMPK activation in the adrenal cortex can act as a direct activator for StAR production in the adrenal.

In addition to inflammatory, immune, and hypoglycemic stress, ACTH does not appear to be the only factor in regulating cortisol release during hypoxemic stress (decreased oxygen in the blood) (10,11). The mechanism for adrenal detection of hypoxia is currently unknown. It has been found that actions similar to that of IL-6 in many tissues can be accomplished by activation of AMPK (12). It has even been demonstrated in a few tissues that IL-6 action can be mediated directly by activation of AMPK (13, 14, 15, 16). AMPK is an energy sensing enzyme that is activated when the intracellular ratio AMP/ATP is elevated. A lack of oxygen in the blood would reduce cellular respiration causing the ratio of AMP to ATP to rise and allow AMPK to act as a sensor for hypoxia. There is some evidence that exposure of bovine adrenocortical tissue to 5-aminoimidazole-4-carboxamide 1-b-D-ribofuranoside (AICAR), an AMPK activator, increases the expression of StAR. From this information we look to AMPK to possibly serve as the link between hypoxia and the release of glucocorticoids.

In order to make certain that AICAR was acting on AMPK we used the AMPK inhibitor compound C. Compound C has unfortunately been found to have some broad effects on

inhibiting cytokine production, inhibiting glycolysis, and affecting other areas like AP-1 action (17, 18). However this is the best compound available to inhibit AMPK. We will also use compound C to determine AMPK's relationship with IL-6.

MATERIALS AND METHODS

MEDIA

DMEM/F12

The DMEM/F12 was purchased from GIBCO (cat# 11330-032). It was supplemented with Cosmic Calf Serum from HyClone (SH300087.04), 10,000 IU/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate from GIBCO (15140-122) diluted 20 times, and insulin-transferrin-sodium-selenite (ITS). To complete 500 ml of DMEM/F12 medium 26.5 ml of Cosmic Calf Serum, 150 µl of penicillin-streptomycin mix (8 ml dH2O with 200 mg streptomycin sulfate, 500 mg penicillin G, 400 mg gentamycin, and 15 mg amphotericin B), and 530 µl of ITS were added. Completion of medium was done as needed and only 500 ml were completed at one time. The complete and incomplete DMEM/F12 were stored in the refrigerator at a temperature of 0º - 4º C.

RPMI

RPMI Medium 1640 was purchased from GIBCO (31800-089) in powder form. To make 500 ml of RPMI medium, 5.20 g of powder were dissolved in sterile water using a stir bar at room temperature. To this solution was added 1.0 g $NAHCO_3$. The pH of the medium was then adjusted to 7.2 using NaOH and HCl. The pH was adjusted slightly lower than desired as per the instructions that came with the RPMI, which indicated that the pH of the solution would go up 0.1-0.3 pH units upon filtration. After adjusting the pH, the RPMI medium was filter sterilized using a vacuum filter in a sterile hood. The RPMI Medium 1640 was stored in a refrigerator at a temperature of 0º - 4º C.

H295R CULTURED CELLS

H295R cultured cells (American Type Culture Collection, Manassas, VA) were incubated in DMEM/F12 complete medium. Five ml of DMEM/F12 complete was added to every 100 mm culture plate. The H295R cells were grown on plates in an incubator set to 36.9º C and 5% CO_2. Cells were treated when they were approximately 75% confluent.

BOVINE ADRENAL ZONA FASCICULATA FRAGMENTS

Bovine adrenal glands were collected from the Deseret Meat slaughterhouse in Spanish Fork. Adrenals were cut out of the cow shortly after it was killed and placed in 1X PBS on ice. Samples were brought back to the lab and were sliced and the zona fasciculata (ZF) was dissected out from the rest of the cortex. Once separated, the ZF was cut into small fragments and placed in RPMI and placed in a shaking water bath at 37º C with oxygen for 30 min to equilibrate before getting treated. Time from death of the cow to treatment was usually 2 to 2 ½ hours and never more than 4 ½ hours.

TREATMENTS

Bovine Time Course

After equilibrating in RPMI at 37º C, half of the tissue fragments were treated with AICAR at 1mM concentration in RPMI. The other half were given a new round of RPMI without AICAR. One sample of each type was taken out of the treatment solution and frozen after 30 min, 60 min, and 180 min of treatment. All samples were given oxygen during the whole treatment period. Each sample contained ~100mg of tissue.

Bovine Dose Response

After equilibrating in RPMI at 37º C samples were treated for 1 hour with varying doses of AICAR dissolved in 2 ml RPMI medium. One sample was given only RPMI medium and

samples 2-6 were given AICAR in the following doses: 0.1 mM, 0.3 mM, 0.5 mM, 1.0 mM, and 3.0 mM. All samples were given oxygen during the whole treatment period. Each sample contained ~100mg of tissue.

Experiments with AICAR and Compound C

These experiments were performed using bovine adrenal tissue fragments. After equilibrating in RPMI at 37º C, half the samples were treated for 30 min with RPMI with 1% DMSO while the other half were treated with 1 mM compound C dissolved in DMSO and added to RPMI to make a final concentration 10 µM compound C in 1% DMSO RPMI. After this pre treatment with compound C, samples were treated with either RPMI with 1% DMSO only, 1mM of AICAR dissolved in RPMI medium with 1% DMSO, compound C as described above, or both AICAR and compound C in RPMI with 1% DMSO. All samples were given oxygen during the whole treatment period. Each sample contained ~100mg of tissue.

Luciferase Reporter Gene Assay

Cultured H295R cells were transfected with 4 µg of the luciferase gene containing plasmid (pGl2), or 12 µg of a 6456 base pair pGL2 plasmid modified to contain the StAR promoter region (-858bp), and 7 µg of a β-galactosidase expression plasmid as an indicator of transfection efficiency.

Lipofectamine-PLUS Reagents (Invitrogen, Carlsbad, CA) were used to transfect the cells according to instructions given by manufacturer. A 12-well plate was seeded with 1.25 x 10^5 cells and incubated 18-24 hours with complete DMEM/F12 media. A solution of 0.6% PLUS reagent and plasmid DNA was incubated in DMEM/F12 supplemented with only ITS for each plasmid for 15 minutes at room temperature together with a 0.6% Lipofectamine solution. While this complete transfection solution was incubating, 400 µl of DMEM/F12 + ITS was

added to each well. After its incubation, the transfection mix was added in 100 µl aliquots to each well and incubated for 5-6 hours. This solution was removed and 1ml of 2X Serum DMEM/F12 media supplemented with Nu Serum only was added to each well and cells were incubated for 18-24 hours. Media was then exchanged for DMEM/F12 + ITS to incubate for 15 – 30 min. Cells were then treated with either a variable dose of AICAR dissolved in DMEM/F12 (Control, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM) for 60 min or with 1mM of AICAR for variable amount of time (30 min, 60 min, and 120 min) for both control and AICAR treated. Each group included the following controls: cells transfected with the basic plasmid treated with DMEM/F12 alone, cells transfected with StAR plasmids treated with DMEM/F12 alone, and cells transfected with β-gal plasmids treated with DMEM/F12 alone. At the completion of treatment, cells were rinsed with cold PBS then 75 µl of cell extraction solution (83 mM K_2HPO_4, 16.63 mM KH_2PO_4) was added to each well. Cells were scraped into a micro centrifuge tube and centrifuged at 12,000 G for 10 min to pellet debris. Luciferase measurement is explained below.

ANALYSES

RT-PCR

mRNA Extraction

Bovine ZF tissue fragments were treated as described above and mRNA was extracted from these fragments using TRI Reagent (Sigma Aldrich T9424). Tissue was placed in a glass homogenizer with 0.5 ml of TRI and homogenized. The mixture was poured into a 1.5 ml centrifuge tube and the homogenizer was rinsed with another 0.5 ml of TRI and this was added to the same centrifuge tube. This solution was incubated for 5 min at room temp to allow complete breakdown of nucleoprotein complex. 0.2 ml chloroform was added to each sample and each was inverted and shaken. All samples were then centrifuged at 12,000 G for 20 min.

After centrifuging, the top aqueous layer was removed and placed into new 1.5 ml centrifuge tubes, and the other layers were discarded. The mRNA was then precipitated by adding 0.5 ml isopropanol and inverting and shaking. Samples were left at room temp for 10 minutes to allow precipitation reaction to progress. Samples were then centrifuged at 12,000 G for 15 min. Following centrifuging, the supernatant was discarded, and 1 ml 75% ethanol was added to each tube. After shaking each tube vigorously they were centrifuged at 7500 G for 5 min. Following centrifuging, supernatant was poured out and samples were allowed to air dry on ice between 5 and 10 minutes. Resulting mRNA pellets were dissolved in 50 µl of nuclease-free water (NFW), and warmed in a 60º water bath for 15 min.

mRNA Quantification

Concentration was quantified using the ND-1000 spectrophotometer from Nanodrop. Using NFW as a blank, 2 µl of each sample was placed on the Nanodrop which determined the concentration of mRNA. Samples were diluted to obtain a working concentration between 1000 and 2000 µg/ml.

Reverse Transcription Reaction

A grand mixture was made that contained the exact ratio of the following: 1.0 µl 10x Buffer, 1.0 µl dithiothreitol (DTT), 0.5 µl Superscript II (reverse transcriptase), 0.5 µl dNTP (deoxynucleotide triphosphates), 0.5 µl RNAse inhibitor, and 0.2 µl 10x decamer. This ratio was multiplied by the number of samples being tested plus one. This grand mixture was combined in a separate microcentrifuge tube, and 3.7 µl of the grand mixture was added to each sample tube. 2 µg of sample mRNA were added to each tube that now contained the grand mixture. NFW was then added to bring each mixture to 10 µl total volume. The tubes were then vortexed,

centrifuged, and placed in the thermocycler (Gene Amp PCR system 2400 Perkin Elmer, Waltham, MA). Thermocycler was run at 42º C for 1 hour.

Polymerase Chain Reaction

A grand mixture was made for the completed RT samples with the following ratios of solutions multiplied by the number of samples plus one: 19.9875 µl NFW, 2.5 µl 10x buffer, 0.3125 µl dNTP, 1.0 µl primer, 0.2 µl JStaq (DNA polymerase). A different grand mixture was made for each primer (one for 18s control and one for the experimental primer). Aliquots (24 µl) of the grand mixture were added to a microcentrifuge tube for each sample. To each of these was added1 µl of the desired RT sample. After vortexing and centrifuging, the tubes were put in the thermocycler (Gene Amp PCR system 2400 Perkin Elmer, Waltham, MA). Initially the DNA would be denatured at a temperature of 95º C for 3 min. The machine would cycle through the next 3 temperatures 35-55 times. First 95º C for 30 sec to separated the template and new complementary strand, then from 56º-58º C depending on the length of primer for 30 sec to allow the primer and coding strand to anneal, finally to 72º C for 45 sec for optimal polymerase action. At the end of these cycles, the machine would hold at 72º for 3 min to allow completion of polymerase reactions followed by being held indefinitely at 4º C to prevent further reactions until PCR mixture was frozen again.

Electrophoresis

Agarose gels were prepared by adding 0.5 g of agarose to 50 ml of Tris-acetate EDTA electrophoresis buffer (TAE) and microwaving for 1 min. After allowing the solution to cool for 3 min, 2.5 µl of ethydium bromide were added and the solution was swirled to mix. Mixture was poured into gel apparatus, bubbles were removed, a 12 well comb was placed in the gel, and it was allowed to harden. TAE was added to the electrophoresis apparatus until it covered the gel.

To 10 µl of each PCR sample was added 2.5 µl of loading dye, and each sample was then mixed by vortexing. 10 µl of each sample and loading dye mix was then added to each well, and the electrophoresis was run at 80 V for 40 min (sometimes a little more or less depending on the sample being run).

The gel was photographed over transilluminating ultraviolet light, and the bands' spot density was quantified using a computer camera and software (Alpha Innotech Corporation, Fluorchem 8900).

Western Blot

Protein Extraction

Bovine tissue was added to a homogenizer with 400 µl of extraction buffer (4 ml per gram of tissue for 100 mg of tissue). Extraction buffer was made of a stock solution which contained 0.79 g Tris-HCl, 4.6 g mannitol, 0.21 g NaF, 0.22 g sodium pyrophosphate, 0.037 g EDTA, 0.038 g EGTA, 1 ml 100x Triton, 10 ml glycerol, and distilled water to a volume of 100 ml to which was added at the time of use 50 µl of 1 mM PMSF in isopropanol, 0.0154 g of DTT, 0.0156 g of benazamidine, and 0.1 mg soybean trypsin inhibitor. Homogenate was poured into 1.5 ml centrifuge tubes and incubated on ice for 60 min. Then homogenate samples were centrifuged at 10,000 G for 10 minutes. The supernatant was retained and stored in the freezer at -90º C. Protein concentration was determined by Bradford assay.

Electrophoresis

Samples were prepared by combining 10 µl of 4x loading buffer (50 ml 0.5 M Tris-HCl, 30 ml glycerol, 8 g SDS, ddH_2O to 92 ml pH to 6.8 if necessary, and 20 mg bromophenol blue, added 8% β-mercaptoethanol (e.g. 80 µl BME + 920 µl 92% 4x Loading buffer) right before use), and a 30 µl mixture of sample and water that would contain 80 µg total protein. These 40

µl mixtures were loaded into the 10% gels from BIO-RAD (161-1155) and run for ~30 min at 200 V in running buffer that was prepared from commercial 10x buffer concentrate from BIO-RAD (161-0732). After electrophoresis, gel cassettes were opened and the gels removed. The wells were cut away from the body of the gel, and the gel was placed in transfer buffer (26.6 g glycine, 6.06 g Tris-HCl, 400 ml methanol, brought to 2000 ml with distilled water) to soak for ~15 min. All filter papers and fiber pads needed for transfer were soaked in cold transfer buffer for at least 45 min before transfer. After soaking transfer cassettes were assembled to transfer proteins to a polyvinylidene fluoride (PVDF) membrane at 100 V for 60 min. After the transfer, the membrane was stained with Ponceau S in 5% glacial acetic acid. This stained all proteins red and allowed us to assess the equivalence of our protein loading for each sample.

Immunoblotting

After rinsing membranes in TBST (2.42 g Tris-HCl, 8.00 g NaCl, pH to 7.6 and bring to 1 L with distilled water, and add 1 ml of polyoxyethylene 20 sorbitan monolaurate (Tween)), they were soaked in 5% dry milk/TBST to cover the membrane in non-specific milk protein for 1 hr. After blocking, the membranes were washed twice in TBST for 5 minutes each time. Then the membranes were soaked in primary antibody solution (1 mg% BSA/TBST with 1:750 to 1:5000 ratio of antibody) overnight while covered at 4º C. The next morning the antibody solution was removed and the membranes were rinsed 3 times for 5 min each in TBST. Then membranes were incubated in HRP conjugated secondary antibody solution (1% dry milk/TBST with 1:500 to 1:5000 ratio of antibody) for 60 min. After removing antibody solution, the membrane was washed 3 times in TBST for 5 min each. Membranes were then soaked in detection solution (western blotting luminal reagent, Santa Cruz Biotechnology, sc-2048) for 1-2 min. The membranes were placed in a plastic sheet protector and exposed to photo film (Classic

Blue Autoradiography Film BX from Midsci) in a darkroom for 1-5 min depending on intensity of bands. After processing with development machine, a picture was taken of the developed film and density of bands was analyzed using Fluorchem 8900 software from Alpha Innotech.

Antibodies

Antibodies for StAR, SF-1, and DAX-1 were purchased from Santa Cruz Biotechnology. The antibodies were rabbit anti-human, but they were used for, and bound to, bovine proteins.

Luciferase Report Gene Assay

β-galactosidase activity was measured using the Galacto-Light Plus system (Tropix/Applied Biosystems, Foster City, CA) according to manufacturer's instructions. 100 µl reaction buffer (lactose substrate) added to 2 µ of each cell extract and incubated for 30 min. Then 100 µl of accelerator (Tropix) was added to terminate enzyme activity ad initiate light emission to be measured by the luminometer (TD-20/20 Turner Designs, Sunnyvale, CA). Luciferase production in transfected cells was measured using the Luciferase Assay System (Promega, Madison, WI). 100 µl of luciferase assay reagent containing luciferin substrate was added to 20 µl of cell extract, and light produced from the luciferase reaction was measured by the luminometer. Results were compiled on an Excel spreadsheet by calculating the ration of Luciferase/β-galactosidase light production, listed as relative light units (RLU). Ratios were then normalized to controls that had not received AICAR, and final numbers were given in terms of fold RLU.

RESULTS

In the first series of experiments, the concentration–related effect of AICAR on the expression of StAR protein and mRNA was determined in fragments of bovine adrenal zona fasciculata. StAR protein was increased in bovine ZF tissue fragments by AICAR administration for 60 min in a dose dependent manner from 0.1 mM through 1.0 mM. Whereas, StAR protein was increased in comparison to the control by all doses of AICAR, the StAR protein response reached a plateau between 1 and 3 mM in that the response to 3.0 mM AICAR was not significantly higher than that of 1.0 mM AICAR (FIGURE 1). In the next series of experiments, the relationship between the incubation period of AICAR exposure and StAR protein expression was determined. AICAR (1 mM) caused an increase in StAR protein levels in bovine ZF in a time dependent manner beginning at 30 min and the augmentation of StAR expression continued to increase through 180 min (FIGURE 2).

Similar to the effect on StAR protein, StAR mRNA was increased in bovine ZF tissue fragments by AICAR administration for 60 min in a dose dependent manner from 0.1 mM through 3.0 mM. StAR mRNA was increased in comparison to the control by 0.3 mM and greater concentrations of AICAR (FIGURE 3). Likewise StAR mRNA was increased by 1.0 mM AICAR in a time dependent manner in that AICAR exposure for 30 min significantly increased StAR mRNA expression and the effect at 60 min was significantly greater than the effect at 30 min and the increase in expression at 180 min in turn was significantly greater than the effect at 60 min. (FIGURE 4).

AICAR may increase StAR mRNA expression through induction of StAR mRNA synthesis or though inhibition of mRNA degradation. In order to determine if AICAR was directly altering StAR mRNA synthesis, a human adrenal tumor cells line that is easily

transfected with the StAR promoter coupled to luciferase was utilized. StAR promoter activity was increased in cultured H295R cells in a time dependent manner by administration with 1.0 mM AICAR from 30 min to 120 min (FIGURE 5A). StAR promoter activity was increased in cultured H295R cells by AICAR administration for 60 min in a dose dependent manner from at least 1.0 mM AICAR to 1.5 mM AICAR. StAR promoter activity was not increased from baseline levels with administration of 0.5 mM AICAR, and 2.0 mM AICAR produced a decrease in activity from the level of 1.5 mM AICAR dose, though it was still significantly higher than baseline activity. 1.5 mM AICAR was the maximal dose for StAR promoter activity (FIGURE 5B).

SF-1 is one nuclear factor among many that contributes to increased StAR production (19, 20). Therefore, we were interested in the effects of AICAR on SF-1 expression. SF-1 protein was increased in bovine ZF tissue fragments by AICAR administration for 60 min in a dose dependent manner from 0.1 mM through 1.0 mM AICAR. Whereas SF-1 protein was increased in comparison to the control by all doses of AICAR, the response to 3.0 mM AICAR was not significantly higher than that of 1.0 mM AICAR (FIGURE 6). Similar to the effects of AICAR on StAR protein, 1.0 mM AICAR caused an increase in SF-1 protein levels in bovine ZF tissue fragments in a time dependent manner beginning at 30 min and continuing through 180 min (FIGURE 7).

The nuclear factor DAX-1 inhibits the expression of StAR and steroidogenic enzymes and therefore is often increased when steroidogenesis is inhibited, and decreased when steroidogenesis is stimulated. Therefore we determined the effects of AICAR on the expression of DAX-1 protein and mRNA in the bovine adrenal ZF.

DAX-1 protein was decreased in bovine ZF tissue fragments by AICAR administration for 60 min in a dose dependent manner from 0.5 mM to 1.0 mM AICAR. The first concentration of AICAR to significantly inhibit DAX-1 was 0.5 mM and the reduction reached a maximum at 1.0 mM. (FIGURE 8). The AICAR inhibition of DAX-1 expression was time dependent in that a 30 min incubation with 1.0 mM AICAR administration caused a significant decrease in DAX-1 protein levels in bovine ZF tissue fragments and this inhibition became larger through 180 min (FIGURE 9). The decrease in DAX-1 protein was accompanied by a decrease in DAX-1 mRNA in that AICAR administration for 60 min decreased DAX-1 mRNA in a dose dependent manner from 0.1 mm AICAR through 3.0 mM AICAR in bovine adrenal ZF fragments. (FIGURE 10).

In the next series of experiments, it was determined if the effects of AICAR on StAR protein expression were due to some type of non-specific effect of the treatment or if they were mediated by activation of the AMPK pathway. This was accomplished by exposing fragments of bovine adrenal ZF tissue to compound C, a known inhibitor of AMPK, and then exposing these tissues to AICAR. AICAR induced increases in StAR protein levels in bovine ZF tissue fragments was reduced by pretreatment and coadministration of compound C (FIGURE 11) . Likewise, AICAR induced increase of SF-1 protein expression in bovine ZF tissue fragments was reduced by pretreatment and coadministration of compound C (FIGURE 12). Similar to the effects on StAR and SF-1, the AICAR-induced decrease in DAX-1 protein levels in bovine ZF tissue fragments was reduced by pretreatment and coadministration of compound C (FIGURE 13). All these results provide evidence that the AICAR effects on this tissue are mediated by activation of the AMPK pathway.

In order to determine if IL-6 is increasing the expression of StAR through activation of the AMPK pathway, fragments of bovine adrenal ZF were exposed to IL-6 in the presence or

absence of compound C. As reported previously, IL-6 significantly increased StAR protein expression in fragments of bovine ZF cells (21). This IL-6 induced increase in StAR protein expression in bovine ZF tissue fragments was reduced by pretreatment and coadministration with compound C (FIGURE 14).

DISCUSSION

It has been noted that ACTH is not the only regulator of cortisol release from the ZF. Particularly in situations of stress outside of acute mental stress (e.g., inflammation or other insults to the immune system) there are other signaling agents that elevate cortisol production and release. One of these mediators appears to be IL-6 (3). In other tissues, the effects of IL-6 are mediated at least in part through activation of the AMPK pathway (13, 14, 15, 16). AMPK has always been known as an energy sensor that activates when the AMP: ATP ratio rises (22). AMPK is active in numerous tissues and has been found to do much more than just turn off energy consuming machinery and turn on energy producing machinery (23, 24). In some cases, such as the IL-6 pathway, it has been found to be the mediator in the action of endogenous signaling agents (13, 14, 15, 16). We wanted to see if AMPK activation caused increased StAR in the ZF as an indicator of increased cortisol production. Though AMPK could also be acting independently, we wanted to see if it played some role in the ZF's response to IL-6 as it has been shown to do in other tissues (12).

In this study, we have demonstrated that AICAR increases the expression of StAR protein in a time and concentration dependant manner. Additionally, when compound C was included with AICAR treatment, levels of StAR, SF-1, and DAX-1 protein were almost completely returned to baseline. This made it clear that nearly all of the response produced by AICAR treatment can be attributed to increased AMPK activity. The increase in StAR protein expression is at least in part mediated by AICAR increasing the expression of StAR mRNA in that AICAR increased StAR mRNA in a time and concentration dependent manner. The increase of StAR protein to nearly 2 fold of baseline is quite large. In a similar manner, StAR mRNA and promoter activity levels rise to about 2 fold of baseline. It was telling to note that in

the time course experiments for StAR that the rise in mRNA levels preceded a similar rise further down the time scale for protein levels.

The increased expression of StAR mRNA by AICAR is at least in part mediated through an increased synthesis of the StAR mRNA because AICAR contributed to an increase of StAR promoter activity in a time and concentration dependent manner..

Therefore, activation of AMPK increases the expression of StAR and probably modifies steroidogenesis. The significance of this observation is supported by the observation that when compound C was added to the IL-6 treatment StAR protein levels were brought down from 2 fold of baseline to just 1.2 of baseline. This clearly shows that AMPK plays a downstream role in IL-6 induced increase of StAR protein. The mechanism through which this occurs in the adrenal or other tissues is unknown (13-16).

In addition to the role of AMPK in IL-6 stimulation of adrenal function, the AMPK pathway may also be involved in the modification of steroidogenesis in the adrenal by other cytokines (2, 3, 13, 25, 26). AMPK's role in raising StAR protein gives a possible mechanism for cortisol elevation in response to hypoxia. This would provide a direct route for upregulation of cortisol similar to that of IL-6 raising cortisol directly in response to inflammatory or immune stress (3). It is clear that AMPK activity leads to greater production of StAR. This would likely lead to more cortisol release, and this would provide a mechanism for direct cortisol increase in hypoglycemic situations. This could have implications in the side effects of drugs that act on AMPK like Metformin, and also in contributing to the possible causes of type II diabetes mellitus.

The mechanisn through which AMPK increases StAR production is unknown. However, AMPK increases the expression of SF-1 which increases the expression of StAR and

steroidogenic enzymes in many tissues (5, 19, 20). Similar to the increase of SF-1, a decrease in DAX-1 leads to more expression of steroidogenic enzymes (5). In our results, SF-1 had an even greater increase than that of StAR at about 2.25 fold of baseline at one hour. One interesting feature of the results for SF-1 was that it continued to increase dramatically for 3 hours all the way to 3.5 fold of baseline while StAR did not rise as much after 1 hour. Likewise, DAX-1 protein and mRNA dropped to less than half of baseline in 1 hour and then down to a third of baseline after 3 hours of treatment. This trend is similar to that of continued change in levels ofSF-1. For these to occur without an attendant increase in StAR protein and mRNA levels seems counterintuitive. There may be other factors keeping the amount of StAR in check in light of the continued rise of SF-1and fall of DAX-1. Possibilities include increased mRNA and protein degradation. However, due to the lack of large increase from 60 minutes to 120 minutes in StAR promoter activity there must be something repressing transcription in the first place. This could be an inhibitor that is connected to StAR, cortisol, or SF-1 in a negative feedback loop to prevent excessive steroidogenesis.

FUTURE EXPERIMENTS

The lab will continue to perform similar tests with AICAR, compound C, and IL-6 on H295R cells and then test them for protein and mRNA levels of StAR, SF-1, and DAX-1. This will provide a stronger connection to human adrenals than is given by the bovine data alone. Also more results will be obtained for mRNA for DAX-1 and SF-1 to make sure the mRNA picture matches up with the protein picture for these two nuclear factors. We so far have only obtained IL-6 data for StAR as the results came just before printing this thesis we have yet to receive the same data for SF-1 or DAX-1.

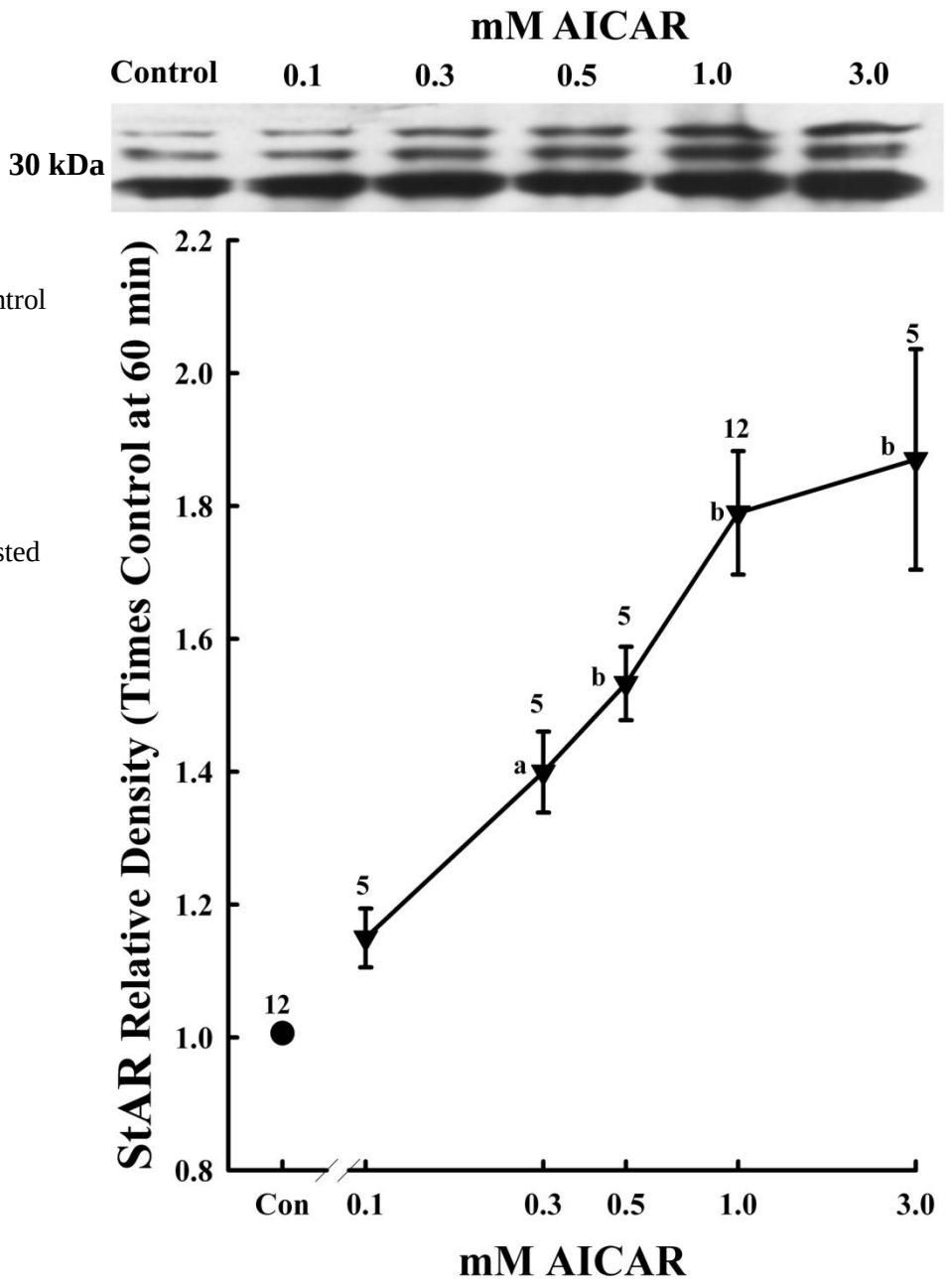
We will also spend some time looking at other steroidogenic enzymes like P450scc, 17α-hydroxylase, 2-hydroxylase, 3-β-hydroxyateroid dehydrogenase, and 11β-hydroxylase. This along with a radioimmunoassay to test cortisol levels will give us a more complete picture of AMPK's influence on steroidogenesis.

From here we will also want to see what AMPK does in the ZR. Steroidogenesis in the ZR was found to be reduced by IL-6. This is likely because the body does not want to dedicate resources to reproductive activities when under immune stress. We suspect the same is likely to be the case with hypoxia and hypoglycemia.

CONCLUSION

Cortisol is an important hormone with numerous roles in the body. Too much or too little cortisol can be very detrimental to normal function of the body. AMPK plays a significant role in the production of cortisol by causing an increase of StAR protein in the zona fasciculata of the adrenal cortex. This may be in response to hypoxia or hypoglycemia, both of which are situations known to cause increased cortisol. Also, AMPK acts as an intermediary for IL-6 in its elevation of StAR in the ZF.

FIGURE 1



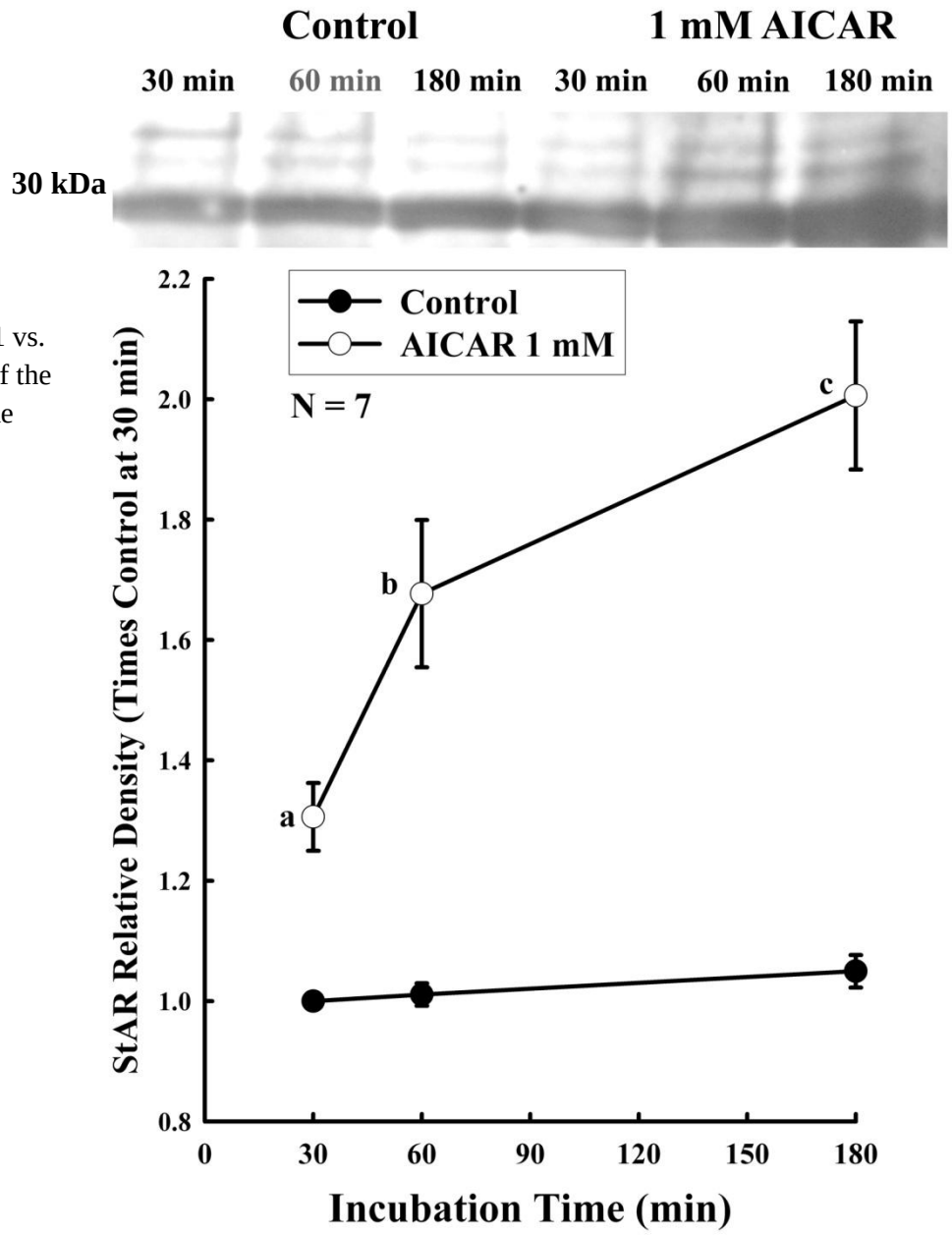
a – $p < 0.05$ vs. control

b – $p < 0.01$ vs. control

5, 12 – number of individual samples tested under each value

Figure 1: AICAR induces increased StAR protein levels in dose dependant manner
Bovine adrenal ZF tissue fragments were treated for 1 hour with increasing dosage of AICAR dissolved in RPMI at 37º C. Following protein extraction, StAR protein levels were determined using western blot. Primary StAR Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 2



a, b, c, – $p < 0.01$ vs. control of the same time point

Figure 2: AICAR induces increased StAR protein levels in time dependant manner
Bovine adrenal ZF tissue fragments were treated with RPMI only or 1 mM AICAR dissolved in RPMI at 37º C for increasing lengths of time. Following protein extraction, StAR protein levels were determined using western blot. Primary StAR Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 3

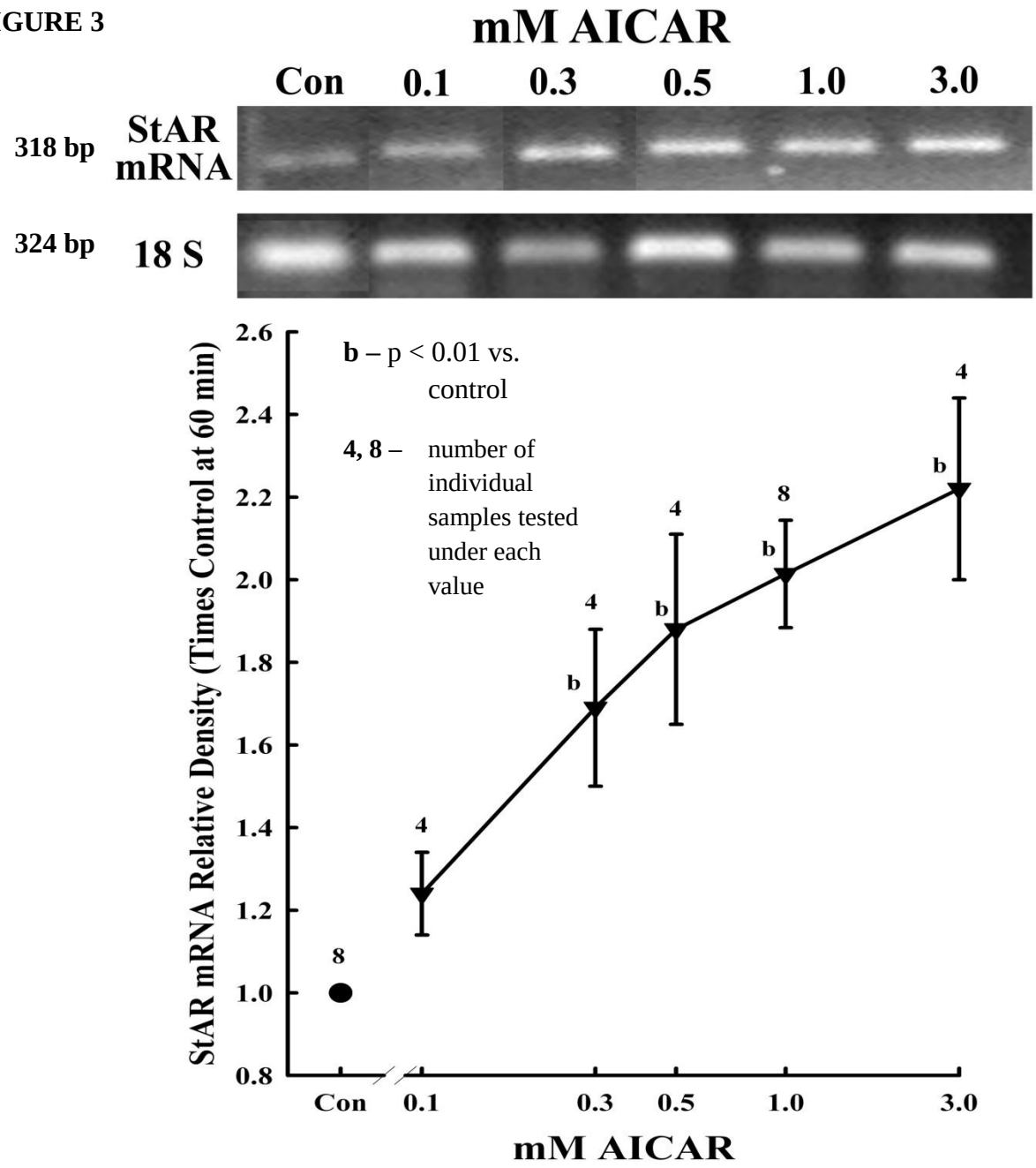


Figure 3: AICAR induces increased StAR mRNA levels in dose dependant manner
Bovine adrenal ZF tissue fragments were treated for 1 hour with increasing dosage of AICAR dissolved in RPMI at 37º C. Following mRNA extraction, StAR mRNA levels were determined using RT-PCR. Custom StAR primers along with standard 18s primers were used in the DNA amplifications. Fold mRNA expression was calculated as a ratio of StAR levels compared 18s levels. Amplified DNA levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 4

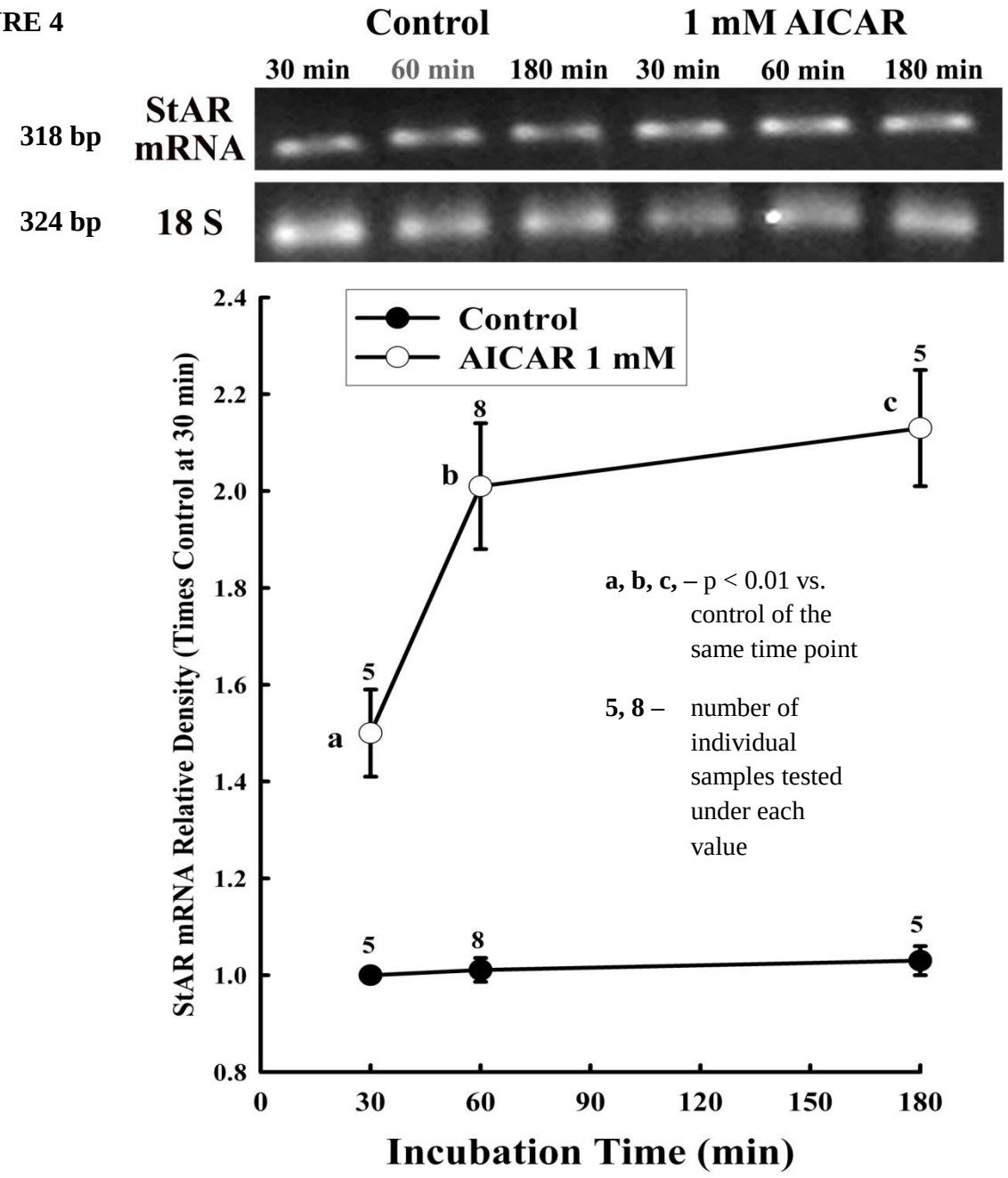


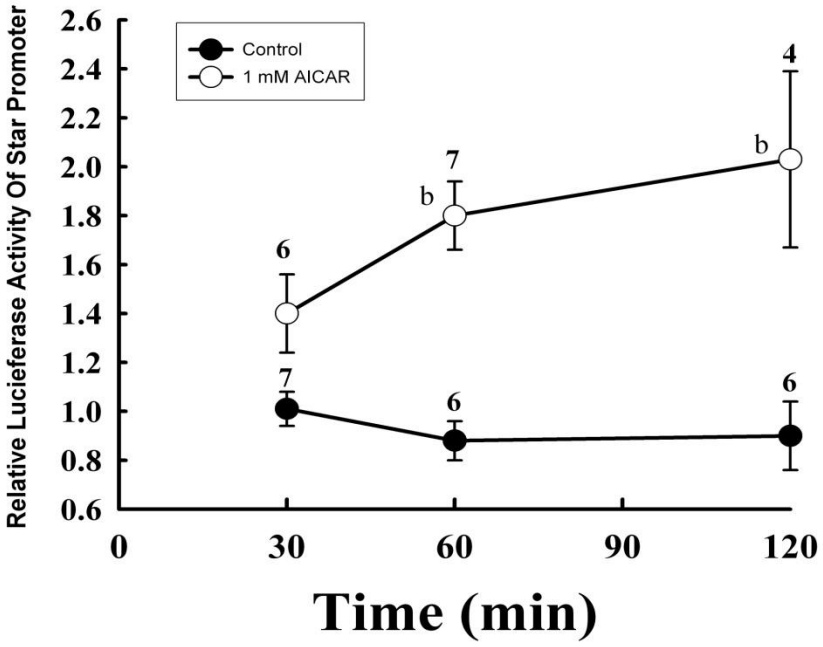
Figure 4: AICAR induces increased StAR mRNA levels in time dependant manner
Bovine adrenal ZF tissue fragments were treated with RPMI only or 1 mM AICAR dissolved in RPMI at 37º C for increasing lengths of time. Following mRNA extraction, StAR mRNA levels were determined using RT-PCR. Custom StAR primers along with standard 18s primers were used in the DNA amplifications. Fold mRNA expression was calculated as a ratio of StAR levels compared 18s levels. Amplified DNA levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 5

A.

b – $p < 0.01$ vs. control of the same time point

4, 6, 7 –number of individual samples tested under each value



B.

a – $p < 0.05$ vs. control

b – $p < 0.01$ vs. control

4 – number of individual samples tested under each value

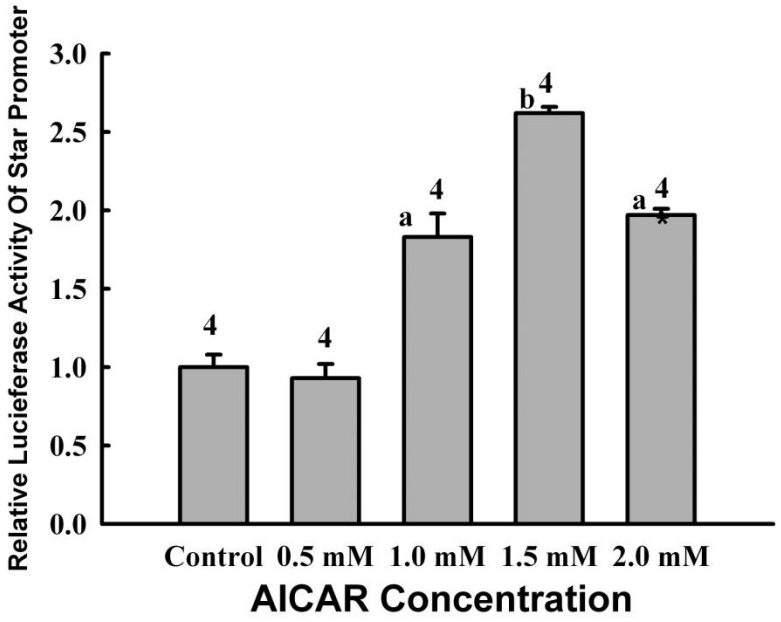
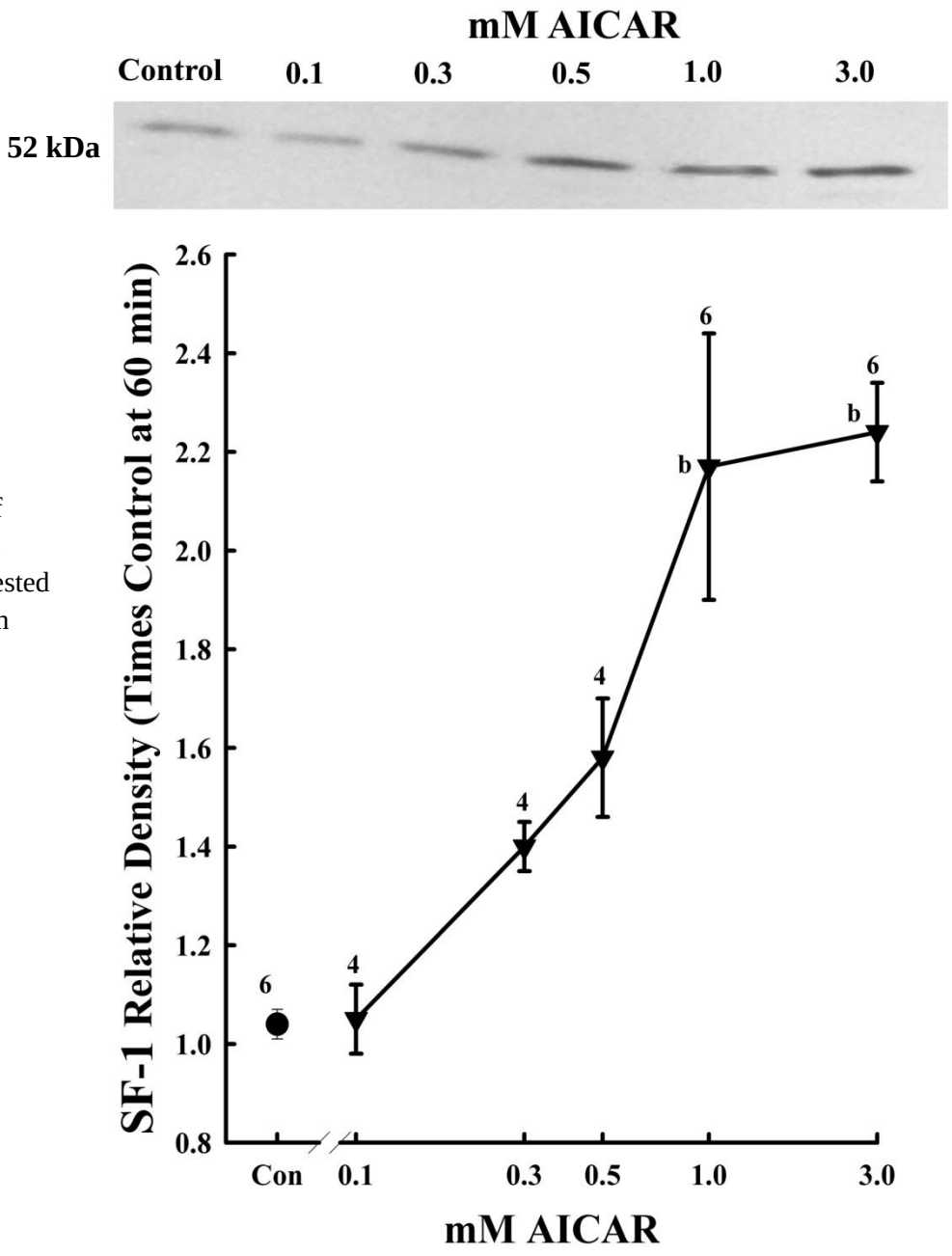


Figure 5: AICAR activates StAR promoter in time and dose dependant manner
H295R cells were transfected with either pGL2 or with both StAR and β-gal plasmids. Cells were then treated with A) DMEM/F12 only or 1mM AICAR in DMEM/F12 for increasing lengths of time B) DMEM/F12 with increasing concentrations of AICAR for 1 hour. Cell treatments were all performed at 37º C and 5% CO_2. RLUs were calculated by use of luciferase assay reagent and Galacto-Plus β-gal detection systems (Tropix) and measured using a TD-20/20 luminometer (Turner Designs).

FIGURE 6

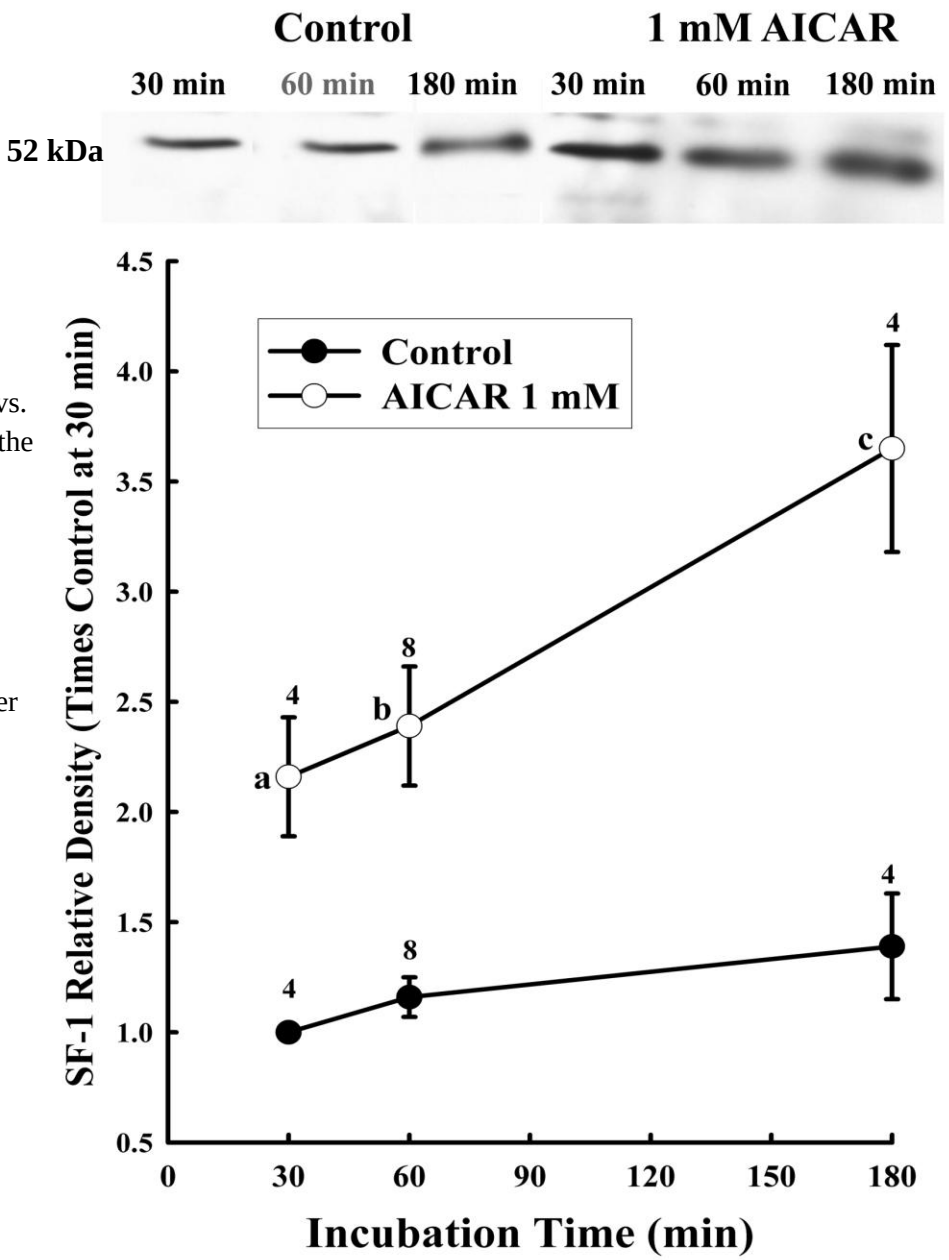


b – $p < 0.01$ vs. control

4, 6 – number of individual samples tested under each value

Figure 6: AICAR induces increased SF-1 protein levels in dose dependant manner
Bovine adrenal ZF tissue fragments were treated for 1 hour with increasing dosage of AICAR dissolved in RPMI at 37º C. Following protein extraction, SF-1 protein levels were determined using western blot. Primary SF-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 7



a, b, c, – $p < 0.01$ vs. control of the same time point

4, 8 – number of individual samples tested under each value

Figure 7: AICAR induces increased SF-1 protein levels in time dependant manner
Bovine adrenal ZF tissue fragments were treated with RPMI only or 1 mM AICAR dissolved in RPMI at 37º C for increasing lengths of time. Following protein extraction, SF-1 protein levels were determined using western blot. Primary SF-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 8

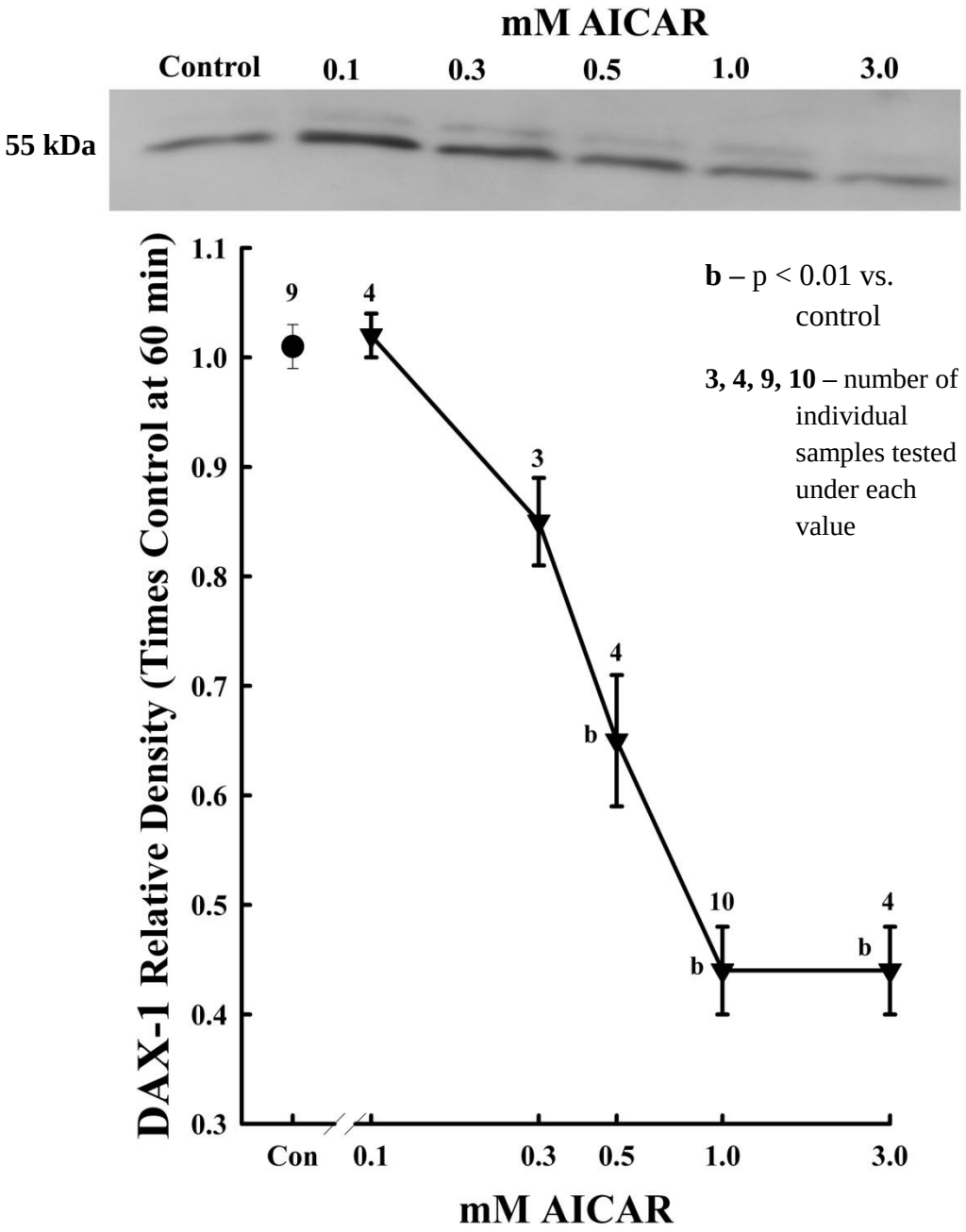
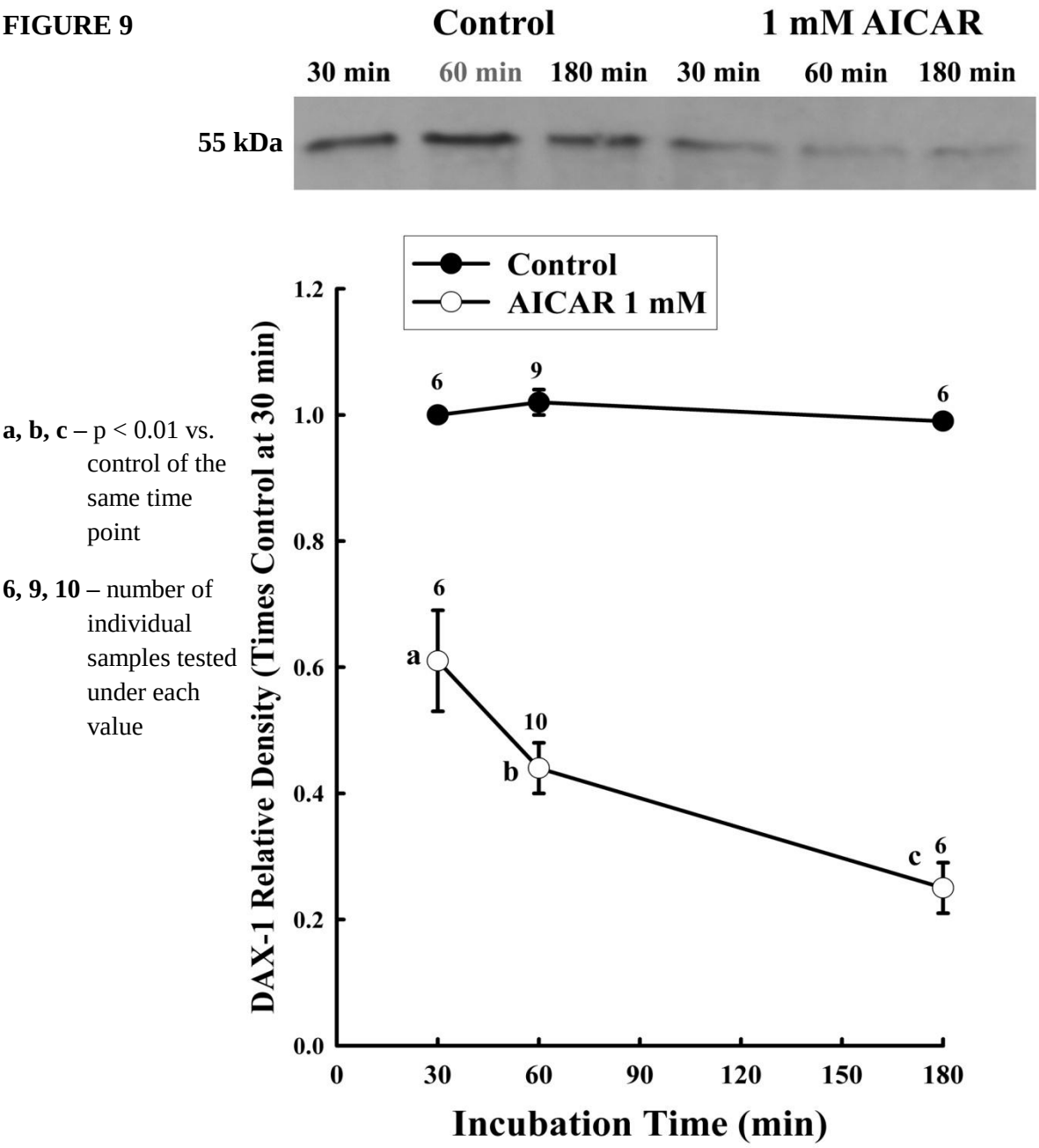


Figure 8: AICAR decreases DAX-1 protein levels in dose dependant manner
Bovine adrenal ZF tissue fragments were treated for 1 hour with increasing dosage of AICAR dissolved in RPMI at 37º C. Following protein extraction, DAX-1 protein levels were determined using western blot. Primary DAX-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 9



a, b, c – $p < 0.01$ vs. control of the same time point

6, 9, 10 – number of individual samples tested under each value

Figure 9: AICAR decreases DAX-1 protein levels in time dependant manner
Bovine adrenal ZF tissue fragments were treated with RPMI only or 1 mM AICAR dissolved in RPMI at 37º C for increasing lengths of time. Following protein extraction, DAX-1 protein levels were determined using western blot. Primary DAX-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 10

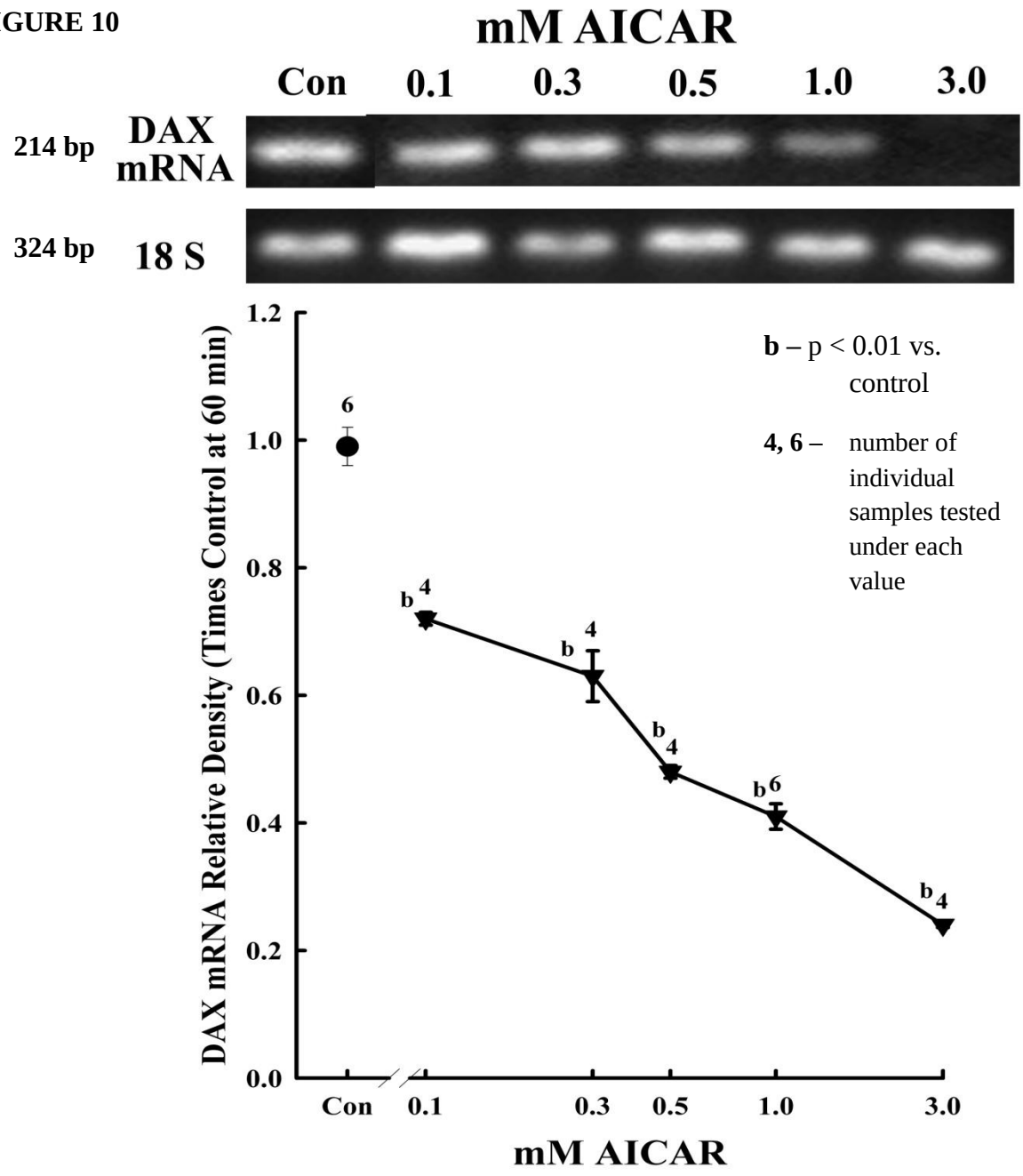


Figure 10: AICAR decreases DAX-1 mRNA levels in dose dependant manner
Bovine adrenal ZF tissue fragments were treated for 1 hour with increasing dosage of AICAR dissolved in RPMI at 37º C. Following mRNA extraction, DAX-1 mRNA levels were determined using RT-PCR. Custom DAX-1 primers along with standard 18s primers were used in the DNA amplifications. Fold mRNA expression was calculated as a ratio of DAX-1 levels compared 18s levels. Amplified DNA levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 11

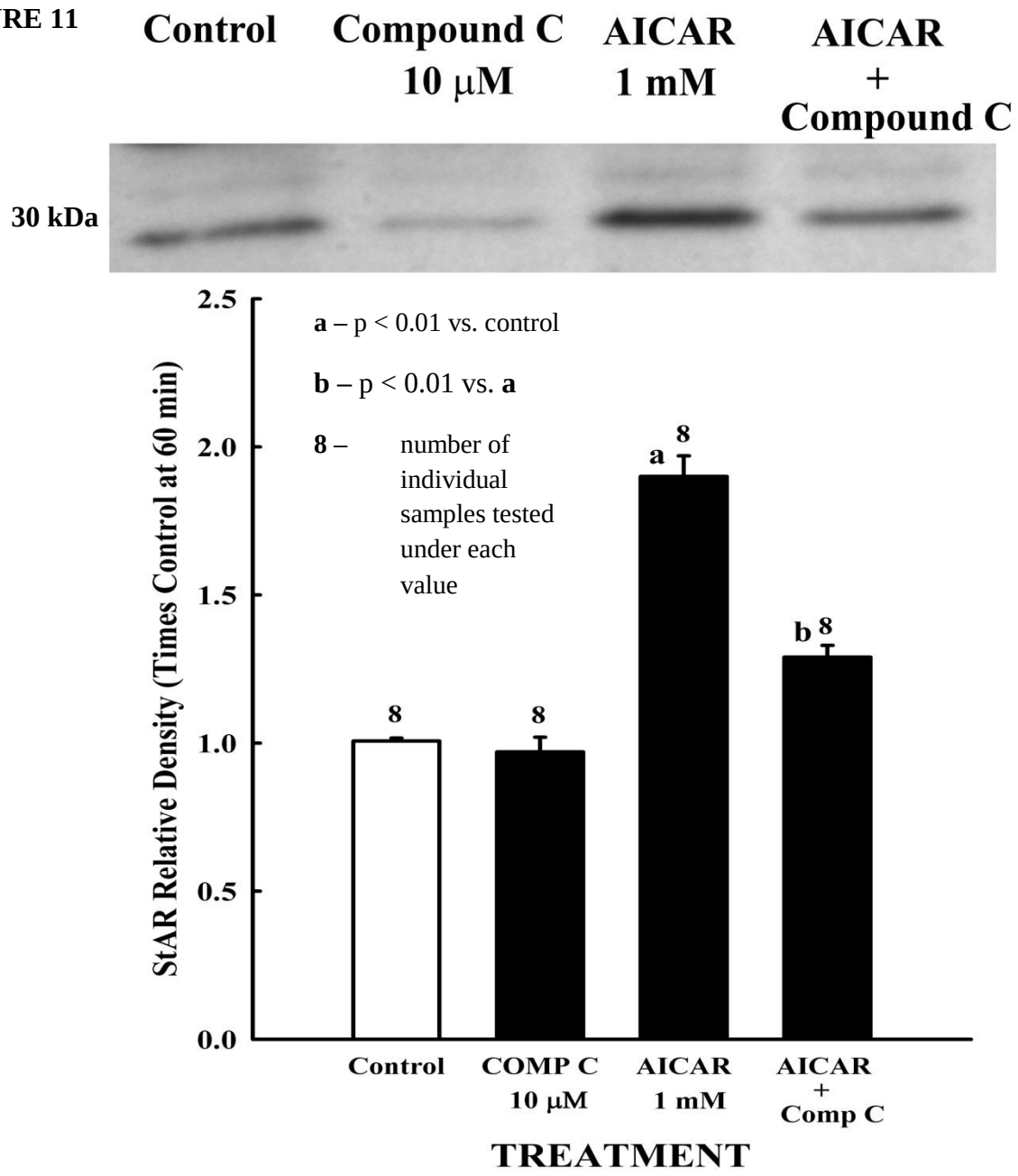


Figure 11: Compound C inhibits AICAR induced increase in StAR protein levels
Bovine adrenal ZF tissue fragments were treated for 1 hour with RPMI only, 10 μM Compound C, 1 mM AICAR, or both together dissolved in RPMI at 37º C. Following protein extraction, StAR protein levels were determined using western blot. Primary StAR Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 12

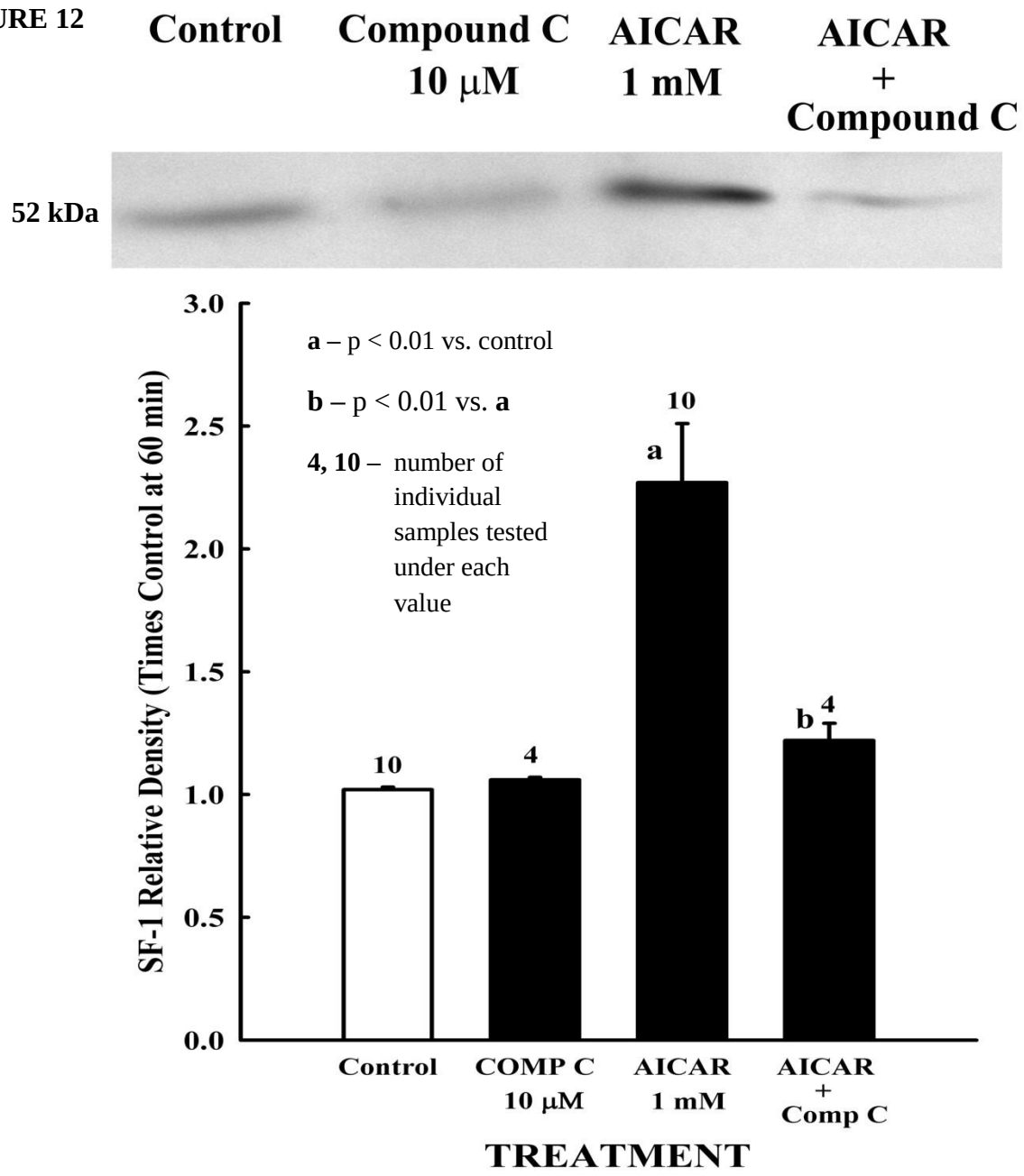
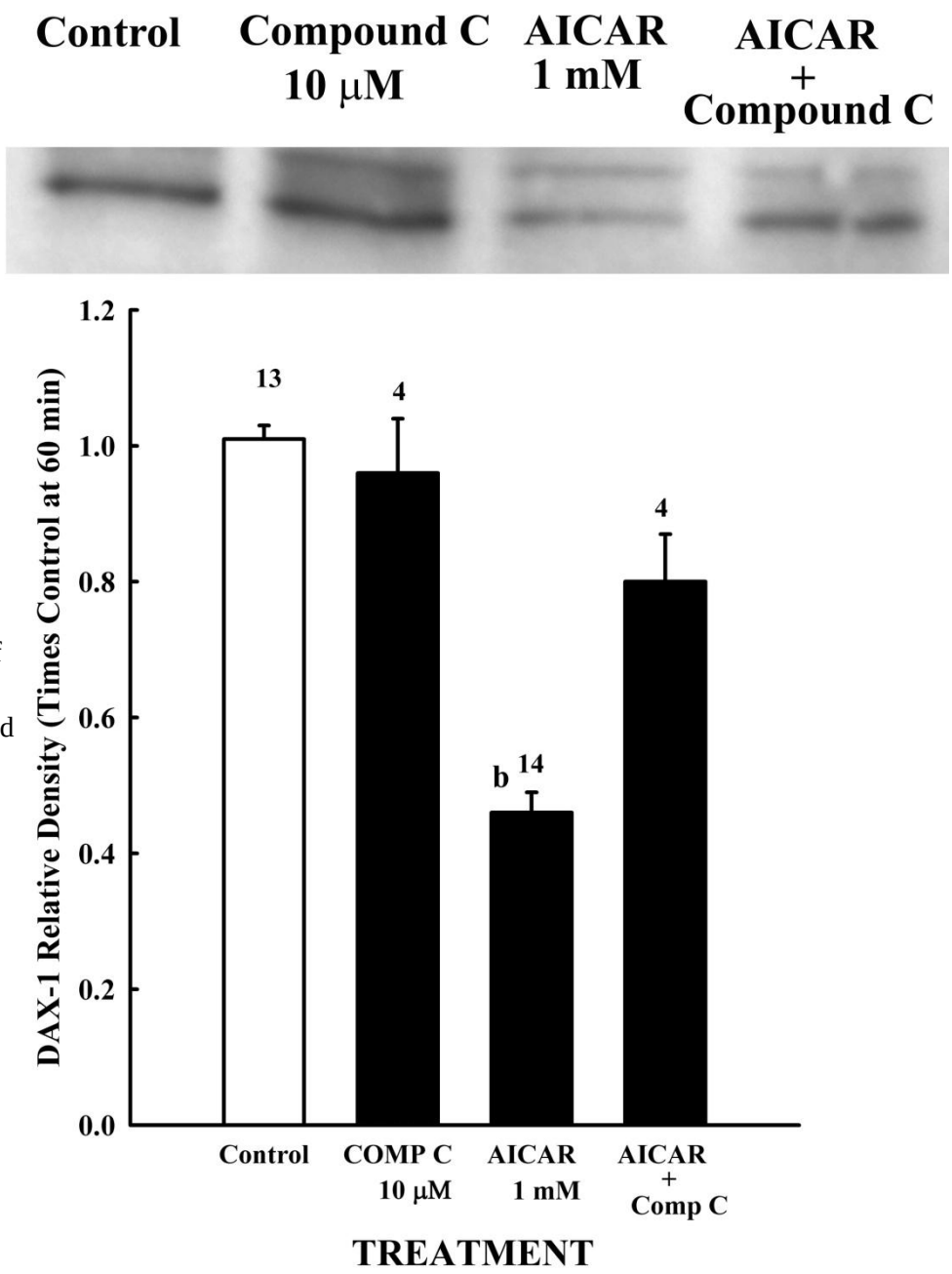


Figure 12: Compound C inhibits AICAR induced increase in SF-1 protein levels
Bovine adrenal ZF tissue fragments were treated for 1 hour with RPMI only, 10 μM Compound C, 1 mM AICAR, or both together dissolved in RPMI at 37º C. Following protein extraction, SF-1 protein levels were determined using western blot. Primary SF-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 13

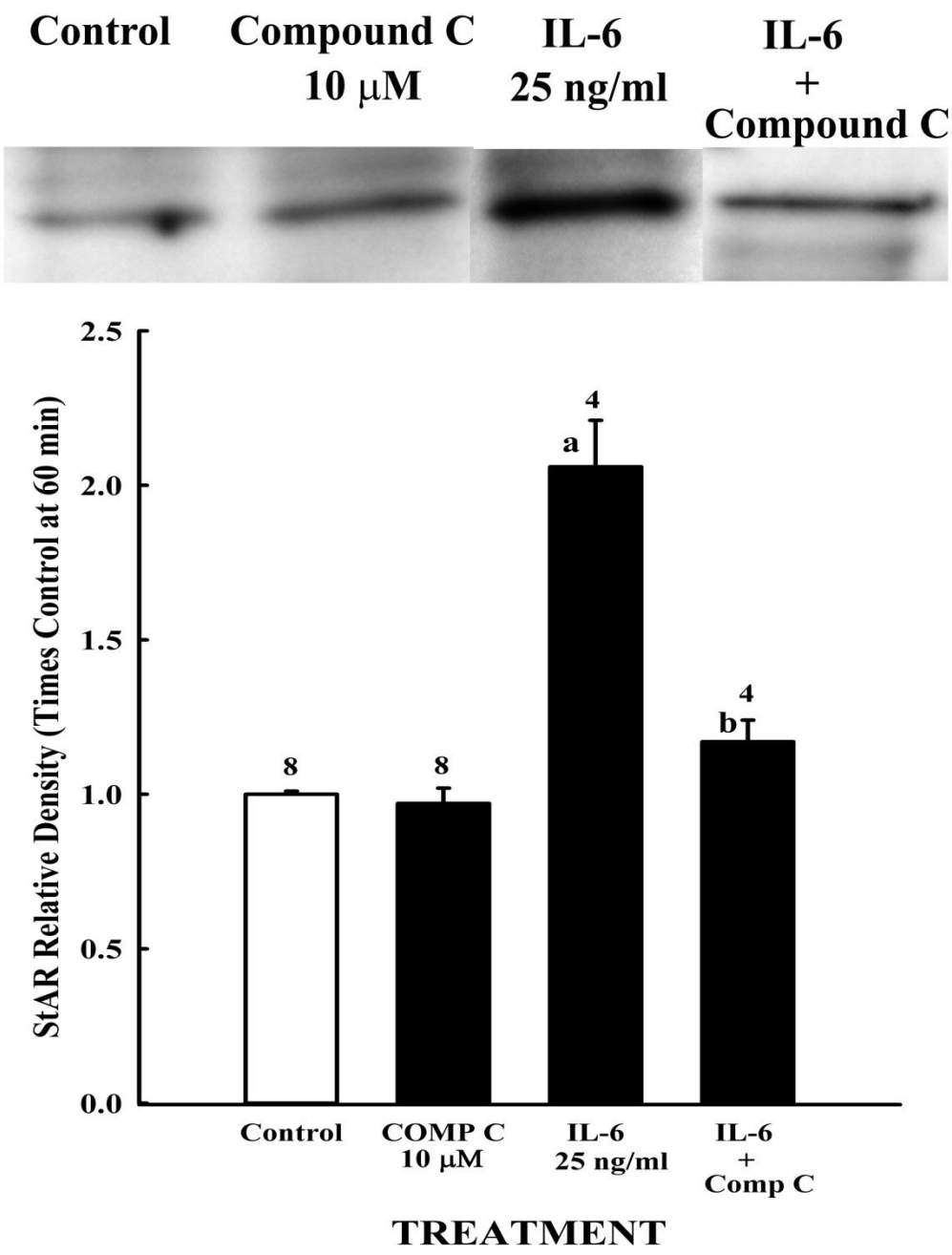


b – $p < 0.01$ vs. control

4, 13, 14 – number of individual samples tested under each value

Figure 13: Compound C inhibits AICAR induced decrease in DAX-1 protein levels
Bovine adrenal ZF tissue fragments were treated for 1 hour with RPMI only, 10 μM Compound C, 1 mM AICAR, or both together dissolved in RPMI at 37º C. Following protein extraction, DAX-1 protein levels were determined using western blot. Primary DAX-1 Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

FIGURE 14



a – $p < 0.01$ vs. control

b – $p < 0.01$ vs. **a**

4, 8 – number of individual samples tested under each value

Figure 14: Compound C inhibits IL-6 induced increase in StAR protein levels
Bovine adrenal ZF tissue fragments were treated for 1 hour with RPMI only, 10 μM Compound C, 25 ng/ml IL-6, or both together dissolved in RPMI at 37º C. Following protein extraction, StAR protein levels were determined using western blot. Primary StAR Antibodies (Santa Cruz) were used. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the spot density tool on the FluorChem 8900 Imaging System and each band was adjusted for local background.

Table 1: Bovine primers used for RT-PCR

Gene	Sequence	Strand
Bovine StAR	CCTCTCTACAGCGACCAA	Sense
	TCGTGAGTGATGACCGTG	Anti-sense
Bovine DAX-1	AGGGGACCGTGCTCTTTAAC	Sense
	ATGATGGGCCTGAAGAACAG	Anti-sense

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Advisor: Dr. Allan M. Judd

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Minor: Business Management

WORK EXPERIENCE

Brigham Young University
Lab Instructor of Advanced Physiology Lab **2008-2010**

Assist students in hands on physiology lessons; grade lab reports and calculate final grades

Cephalon, Inc.
Quality Control Chemist **2007-2008**

Chemically tested raw materials and finished pharmaceutical products for compliance with specifications; wrote and edited standard operating procedures, and updated testing methods; attended European Pharmacopeia conference on behalf of my department, returned and reported

Brigham Young University
Teaching Assistant and Tutor **2005-2007**

Taught recitation classes for genetics and general biology; developed and demonstrated practice problems in general lecture of 800 students; tutored student athletes in various biology, physics, and chemistry classes

Step Beyond, Inc.
Youth Tracker **2004-2005**

Worked with troubled youth to set up school, work, restitution hours, and therapy; took youth shopping weekly and gave rides when needed; performed long distance transfers to place youth in homes

POSTERS PRESENTED

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Presented at the Annual Endocrine Society Conference, San Diego, CA **2010**

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Spanish – speak fluently and read/write with high proficiency

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Physiology and Developmental Biology Department **2008 -2010**