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Involvement of AMPK and AP-1 Biochemical Pathways in IL-6 Regulation of Steroidogenic Enzymes in the Adrenal Cortex

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ABSTRACT

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The adrenal cortex is a crucial endocrine gland in the mammalian stress response. In chronic inflammatory stress, cortisol is elevated whereas adrenal androgens are decreased. Furthermore, ACTH levels have poor correlation with the plasma cortisol in these conditions, thus suggesting that other factors are driving the stress response during chronic inflammatory stress. Interleukin-6 (IL-6), a cytokine which is released during chronic inflammatory stress, is assumed to be one such factor. Thus the biochemical pathways by which IL-6 increases cortisol release from the zona fasciculata (ZF), and decreases adrenal androgen release from the zona reticularis (ZR) were investigated.

Since IL-6 activates AMP-activated kinase (AMPK) in skeletal muscle, AMPK was investigated for IL-6- induced effects in ZF and ZR tissue. The effects of AMPK activation and IL-6 exposure on the expression of the steroidogenic proteins, steroidogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (P450scc), and on the steroidogenic nuclear factors steroidogenic factor-1 (SF-1) and adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-1) were investigated. AMPK activation and IL-6 exposure increased the expression of StAR, P450scc, and SF-1, and decreased DAX-1 in the ZF. Meanwhile, AMPK activation and IL-6 exposure decreased the expression of StAR, P450scc, and SF-1, and increased DAX-1 in the ZR. AMPK inhibition blocked the effects of AMPK activation and IL-6 on the ZF and ZR.

Activator Protein-1 (AP-1) was the second biochemical intermediate studied since in other tissues AMPK activation increases the expression and phosphorylation of AP-1 subunits. IL-6 stimulation and AMPK activation increased the expression of the AP-1 subunits cFOS, cJUN, JUN B, and JUN D, while increasing the phosphorylation of cJUN in both the ZF and the ZR. These effects were blocked by AMPK inhibition. Inhibition of AP-1 leads to decreased StAR, P450scc, and SF-1, and increased DAX-1 in the ZF. Meanwhile, AP-1 inhibition leads to increased StAR, P450scc, SF-1, and decreased DAX-1 in the ZR. Therefore the AP-1 complex functions as a biochemical intermediate in the IL-6 and AMPK regulation of steroidogenic enzymes in the ZF and ZR.

Overall, the results suggest that IL-6 activates AMPK, which increases the expression and phosphorylation of AP-1 subunits in the ZF and the ZR. However, increased AP-1 activation leads to increased StAR and P450scc in the ZF, but decreased StAR and P450scc in the ZR.

Keywords: adrenal cortex, zona fasciculata, zona reticularis, chronic inflammatory stress, interleukin-6, AMP-activated protein kinase, activator protein-1, StAR, P450scc, SF-1, DAX-1.

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CHAPTER 1: REVIEW OF LITERATURE

Hypothalamic-Pituitary-Adrenal (HPA) Axis

A disturbance to an organism's physical or psychological health results in a stress response. The mammalian stress response consists of a complex interplay between the nervous system, endocrine system, and the immune system. The nervous system and the endocrine system coordinate to form the neuroendocrine system which regulates the release of the major stress hormone cortisol, while cortisol regulates the functioning of the immune system to complete the stress response. The stress response allows the organism to maintain homeostatic conditions amidst the stressful state [1, 2, 3, 4]

Classically it is known that during stress the hypothalamic-pituitary-adrenal (HPA) axis is activated resulting in an in elevation in blood cortisol levels [1, 2, 3, 4, 5]. According to the classical HPA axis model, stress stimulates the parvocellular neurons in the paraventricular nucleus of the hypothalamus to release corticotropin-releasing hormone (CRH) and vasopressin (AVP). These neuropeptides (CRH and AVP) enter the hypothalamic-pituitary-portal system and bind to corticotrophs in the anterior pituitary gland to stimulate the production of adrenocorticotrophic hormone (ACTH) from proopiomelanocortin (POMC). The ACTH is then released into the systemic circulation, where it binds to ACTH receptors in the adrenal cortex stimulating the release of steroid hormones, especially cortisol [2, 4].

Cortisol is the primary steroid hormone (glucocorticoid) produced by the zona fasiculata (ZF) of the adrenal cortex [4, 5, 6]. The stimulation of the adrenal cortex by ACTH also results in aldosterone and adrenal androgen production by the zona glomerulosa (ZG) and the zona reticularis (ZR) respectively [2, 4]. However, aldosterone production from the ZG is primarily regulated by plasma potassium concentrations and angiotensin II [1, 2, 3]. Cortisol, aldosterone

and adrenal androgens are vital for the maintenance of normal physiology during stress. Cortisol regulates cellular metabolism, maintains blood glucose levels, and modulates the inflammatory and immune response [2, 8, 15]. Aldosterone primarily maintains the water and salt balance of the body. Adrenal androgens which include dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione, affect skeletal muscle, immune and inflammatory systems, the central nervous system, the cardiovascular system, caloric intake, body composition, and metabolism [1, 2, 3, 4].

Dysregulation of the HPA Axis in Chronic Stress

Although the acute stress response can be explained by the classical HPA axis model, recent studies have demonstrated that chronic forms of stress may regulate adrenal function through other mechanisms [1, 2, 7]. Especially during chronic inflammatory stress, cortisol release does not appear to be stimulated in accordance with the HPA axis. In patients with chronic inflammatory stress, ACTH levels decrease with the transition from the acute phase to the chronic phase, whereas cortisol levels remain elevated throughout the chronic stress phase [2, 5, 9]. Thus, the classical HPA axis does not explain cortisol release during chronic inflammatory stress. However understanding the physiology of chronic stress is vital because major injuries, surgery, inflammatory diseases, and various forms of viral, bacterial, and parasitic infections lead to chronic stress. Since ACTH does not control cortisol release during chronic inflammatory stress, it is suggested that other agents stimulate cortisol release during this condition. Cytokines have been suggested to drive the stress response in chronic stress situations. In particular the cytokine interleukin-6 (IL-6) may stimulate cortisol release during chronic stress [4, 8, 9, 10, 11, 12].

The mechanisms involved in the regulation of adrenal androgens are poorly understood. Although ACTH stimulates adrenal androgen release, there is a poor correlation between plasma concentrations of ACTH and plasma concentrations of adrenal androgens. Adrenal androgens increase during acute stress similar to cortisol. However, during chronic stress the plasma concentrations of adrenal androgens greatly decrease [1, 2, 7, 9]. Although the exact mechanism by which adrenal androgens decrease is not fully understood, various cytokines such as IL-6 may play a role in the decrease of adrenal androgens during chronic stress.

Interleukin-6 (IL-6)

Cytokines are peptides which act as intercellular signaling molecules that are released in large quantities from numerous cell types during inflammation. Since chronic inflammation increases the production of cytokines, these inflammatory cytokines could be acting as signaling molecules which trigger the increased release of cortisol. In particular, IL-6 is known to be elevated during chronic inflammation, and has been shown to regulate cortisol release during chronic inflammatory stress [8, 10, 11, 12, 13].

According to previous studies, the ZF of the bovine adrenal cortex releases IL-6, and IL-6 significantly increases basal and ACTH-stimulated cortisol release in a concentration and time dependent manner [14, 15, 16]. Binding studies and immuno-histochemical analysis have demonstrated that ZF cells have IL-6 receptors [17, 18]. Studies with adrenal cells in primary culture have demonstrated the expression of IL-6 at both the mRNA and protein levels [14, 15]. Incubation of cultured human adrenocortical cells with IL-6 increased the release of cortisol and aldosterone in a time and dose dependent manner. In cultured bovine cells, IL-6 increases basal cortisol release within 8 h and ACTH-stimulated cortisol release within 4 h (both about 100% increases), and these effects persisted through at least 24 h [14, 16]. However in contrast to an

increase in cortisol release from the ZF, in the bovine adrenal ZR IL-6 exposure leads to a decrease in adrenal androgen release [19].

The direct stimulation of the adrenal ZF by IL-6 may explain the high plasma cortisol levels in the absence of an increase in ACTH [14, 19]. Thus, IL-6 is assumed to play a critical role in the regulation of adrenal steroidogenesis in the adrenal cortex. In support of this hypothesis, various experiments in humans indicate that IL-6 may be stimulating the adrenal cortex to release cortisol and inhibit the release of adrenal androgens. Acute injections of recombinant IL-6 to human patients resulted in both a dose-dependent and time-dependent increase in plasma cortisol levels [12]. Furthermore, a long-term human trial with daily subcutaneous injections of IL-6 demonstrated that on day one, the administration of IL-6 resulted in the rapid increase of both ACTH and cortisol concentrations in the plasma. However on day 21, the ACTH response to IL-6 was attenuated, whereas the IL-6-induced cortisol release remained unaffected [20]. This observation suggests that in humans IL-6, in addition to its effects in the hypothalamus and pituitary, may stimulate cortisol release directly at the level of the adrenal gland. Therefore the elevated levels of cortisol in chronic stress in the presence of normalized or low ACTH levels may be maintained by the direct action of IL-6 on the adrenal gland. Moreover the drug tocilizumab, an anti-interleukin-6 receptor antibody, reduces cortisol levels in human patients [21]. This reduction of plasma cortisol concentrations may be a result of the neutralization of IL-6 by the anti-interleukin-6 receptor antibody, which further supports the hypothesis that IL-6 is directly involved in cortisol release.

In support of a role of IL-6 in inhibiting adrenal androgens in humans, anti-interleukin-6 receptor antibody increases the secretion of adrenal androgens in rheumatoid arthritis patients diagnosed with elevated levels of plasma IL-6 [22]. Thus the neutralization of IL-6 with the anti-

interleukin-6 receptor or anti-interleukin-6 antibodies in humans results in a decrease in plasma cortisol and an increase in plasma adrenal androgens. Thus these findings support the hypothesis that IL-6 regulates adrenal steroidogenesis in the ZF and the ZR.

In addition to human studies, a variety of studies in rodents support the role of IL-6 in regulating glucocorticoid release in chronic stress situations. Mice infected with the murine cytomegalovirus showed elevated levels of IL-6, and during the chronic phase of the infection these mice showed increased levels of corticosterone, which is the rodent equivalent of cortisol [23]. Furthermore, mice injected with anti-interleukin-6 receptor antibody had a significant time-dependent decrease in corticosterone release, which could be explained by the antibody's neutralization effect on IL-6 [24]. Thus, IL-6 acts as a regulator of corticosterone release in mice under chronic inflammatory stress.

Although the complete mechanism of IL-6 action in the adrenal cortex is unknown, IL-6 has been demonstrated to modify the expression of several key steroidogenic enzymes and nuclear factors involved in the regulation of expression of steroidogenic enzymes. In the bovine ZF and the human adrenocortical tumor cell line H295R, IL-6 increases StAR (steroidogenic acute regulatory protein) gene transcription and StAR protein expression, associated with increased rates of cortisol release [16]. StAR functions to transport cholesterol from the outer to the inner mitochondrial membrane where the first step in steroidogenesis occurs. This transport of cholesterol is the rate-limiting step in steroidogenesis. Numerous experiments have demonstrated that the rate of cholesterol transport and steroidogensis is directly related to modifications in the expression of StAR mRNA and protein [25, 26, 27, 28]. Therefore agents that increase steroidogenesis in the adrenal gland increase the expression of StAR protein, and an increase in StAR protein expression is indicative of an increase in steroid hormone production

stimulated by IL-6. The cholesterol transported into the mitochondria is cleaved by the enzyme P450scc (cholesterol-side-chain cleavage enzyme) to form pregnenolone. Pregnenolone is then acted upon by several other enzymes to produce cortisol, adrenal androgens, and aldosterone [29, 30]. Therefore the regulation of the expression of P450scc is another key step in the regulation of steroidogenesis.

Various nuclear factors regulate the expression of the steroidogenic enzymes including StAR and P450scc. Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor that binds to a SF-1 binding site localized to the promoters of the genes for many steroidogenic proteins, and in combination with other nuclear factors increases the expression of the mRNA for steroidogenic proteins [31, 32, 33, 34]. The expression of SF-1 is increased by ACTH and other factors that increase steroidogenesis [16]. Inhibition of SF-1 expression by short inhibitory RNAs(siRNAs) or SF-1 activity by a specific SF-1 inhibitor decreases the expression of many steroidogenic enzymes including StAR and P450scc under both stimulated and unstimulated conditions [35, 36]. In contrast, dosage-sensitive sex reversal adrenal hypoplasia congenital, critical region on the X chromosome, gene-1 (DAX-1) is a nuclear factor that inhibits the expression of many steroidogenic proteins. Therefore the expression of DAX-1 is decreased by factors that stimulate steroidogenesis [37, 38, 39, 40, 41, 42, 43].

In many different tissues including the adrenal cortex, IL-6 signals through the Janus kinase / signal transducers and activators of transcription (JAK/STAT) pathway [44, 45, 46]. IL-6 binds to the cell surface receptor gp80, which then leads to the dimerization of two gp130 molecules located on the cell surface. The dimerization of gp130 results in the phosphorylation of the attached Janus kinase 2 (JAK2) thereby activating it. The phosphorylated JAK2 then phosphorylates the cytoplasmic domain of gp130. The phosphorylated gp130 domain acts as a

docking site for STAT1 and STAT3 to be phosphorylated by JAK 2, and translocated into the nucleus [46].

The StAR promoter contains binding sites for STAT1, STAT3, and other nuclear factors. Thus IL-6 could recruit STAT1 and STAT3 to the StAR promoter through the JAK/STAT pathway to induce StAR gene transcription, thereby leading to increased cortisol production [44, 46]. In support of this hypothesis JAK/ STAT inhibitors block the effects of IL-6 on cortisol release (our unpublished observation). However, the STATs recruited to the nucleus as a result of IL-6 signaling could also bind to promoters of other essential steroidogenic nuclear factor genes such as DAX-1, SF-1, and activator protein-1 (AP-1) [46].

AMP-activated Protein Kinase (AMPK)

Activation of other biochemical intermediates such as adenosine-monophosphate-activated protein kinase (AMPK) may also play a role in regulating steroidogenesis [47, 48, 49, 50]. In the ovary, AMPK is expressed and activation of AMPK modifies the production of steroid hormones [48, 49]. AMPK is also present and is activated in the adrenal cortex in response to chronic inflammatory stress [50]. Furthermore, adiponectin in the H295R adrenal tumor cell line increases AMPK activation and cortisol release, and these effects are blocked by the AMPK inhibitor compound C [49]. Preliminary experiments have demonstrated that the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR) leads to increased expression of StAR in the bovine adrenal ZF [16]. Thus AMPK regulates steroidogenesis in the ovary and the adrenal [47, 48, 49, 50]. AMPK may play a role in regulating adrenal steroidogenesis either through activation by the metabolic state of the adrenal gland, or AMPK may be part of the biochemical pathways through which IL-6 modifies steroidogenesis. In

support of this hypothesis, IL-6 activates AMPK in skeletal muscle resulting in alterations in carbohydrate metabolism [51, 52].

Structurally AMPK is a αβγ heterotrimer composed of an α-catalytic subunit and βγ-regulatory subunits, while AMPK subunits show different tissue-specific expression and activation patterns. AMP binds to the enzyme's γ subunit [51]. AMPK is phosphorylated in Thr-172 in the activation loop of the α-subunit catalytic domain by LKB1, an upstream kinase. AMPK plays a key role in the regulation of cellular metabolism, as it acts as the master regulator of protein, lipid and glucose metabolism in response to metabolic stress and energy demands [51]. AMPK integrates stress responses such as exercise as well as nutrient and hormonal signals to control substrate utilization and energy expenditure. AMPK is commonly known to be activated by skeletal muscle contraction in exercise. AMPK activation promotes energy expenditure through upregulation of fatty acid oxidation, glucose uptake, and mitochondrial biogenesis in skeletal muscle [52]. AMPK suppresses hepatic glucose synthesis and lipogenesis in the liver. AMPK regulates cellular energy metabolism through direct effects on gene transcription and metabolic enzymes. It is also shown that AMPK promotes food intake in the brain [51, 53]. In fact, AMPK is an intricate signaling junction in many biochemical pathways.

The compound 5-aminoimidazole-4-carboxamide riboside (AICAR) activates AMPK by passing through the cell membrane. In the cell the AICAR is metabolized to ZMP (AICAR monophosphate) which mimics the effects of AMP on AMPK-kinase, while the cellular levels of AMP, ADP, ATP remain unchanged [51]. AICAR promotes glucose transport into skeletal muscle and inhibits hepatic glucose production. AICAR is commonly known to increase AMPK-dependent glucose uptake through translocating GLUT4 receptors to the cell membrane. AICAR also induces fatty acid oxidation in muscle and inhibits cholesterol synthesis in the liver [51].

However AICAR, through AMPK activation could play different roles in different tissue types such as the adrenal cortex.

Clinical studies have proven that cytokines such as IL-6 are elevated in obesity and metabolic syndrome [51]. IL-6 is produced and released from skeletal muscle during exercise suggesting that IL-6 regulates metabolism [51, 52]. In vitro and in vivo studies in rodents and humans have shown that IL-6 increases fatty acid oxidation and glucose uptake [51]. IL-6 knockout mice show significantly low AMPK activation in the liver, adipose tissue, and skeletal muscle [51]. These evidences suggest a functional link between IL-6 and AMPK, which could also be true in the adrenal cortex.

Activator Protein-1 (AP-1)

Activator protein-1(AP-1) is a nuclear transcription factor complex consisting of homo or hetero dimers of JUN and FOS family proteins. The three Jun family members (cJUN, JUN B, and JUN D) and the four FOS family members (cFOS, FOSB, FRA-1, and FRA-2) can combine to form 18 different dimeric combinations of AP-1 [54, 55]. The AP-1 nuclear transcription factor activates steroidogenic genes, including the genes for StAR and P450scc [56, 57]. Furthermore, IL-6 increases the expression of AP-1 proteins in other tissues [57, 58]. Therefore it can be proposed that the STATs recruited to the nucleus through IL-6 induced JAK/STAT signaling binds to the promoters of the AP-1 subunit genes. STAT binding, together with interactions with other biochemical pathways such as AMPK may increase the transcription of AP-1 proteins. In support of a possible role of AMPK in the induction of expression of AP-1 proteins, AMPK activation in a pituitary tumor cell line increases binding of the AP-1 complex to the promoter of the POMC gene [59]. The increased expression of AP-1 is hypothesized to

cause increased activation of the promoters of the steroid hormone genes thereby increasing the expression of steroidogenic proteins.

Hypothesis

In this study I shall investigate the biochemical pathways through which IL-6 modifies adrenal steroidogenic enzyme expression in the bovine ZF and the ZR. It is hypothesized that IL-6 activates AMPK, which in turn leads to the increased expression of AP-1 proteins. The up-regulation of AP-1 subunit expression in turn is hypothesized to regulate the expression of StAR and P450scc proteins, and the nuclear factors SF-1 and DAX-1.

CHAPTER 2: MATERIALS AND METHODS

RPMI Medium Preparation

RPMI 1640 medium was prepared on the day of each experiment. RPMI 1640 medium was obtained from GIBCO, Life Technologies, Grand Island, NY, USA. The powdered RPMI medium was dissolved in distilled water at room temperature with 2 g per 1000 ml $NaHCO_3$ powder as directed by the manufacturer. The pH of the medium was adjusted to 7.4 using 1M NaOH and HCl solutions.

Isolation of Adrenal Tissue

Bovine adrenal glands were used to obtain the zona fasciculata (ZF) and zona reticularis (ZR) tissues for all the experiments. The adrenal glands utilized in all experiments were isolated from adult cows of mixed breeds (beef and dairy) that were sold to two local abattoirs (Deseret Meat, Spanish Fork, UT, USA or Dale T. Smith and Sons Inc., Draper, UT, USA) because of various conditions including age, poor milk production, failure to get pregnant, or thinning of herds. Bovine adrenal glands were collected from the carcasses of animals that had been sacrificed about five minutes previously. The adrenal glands were immediately incubated in 1×PBS (Phosphate Buffered Saline) at 4°C, and transported to the laboratory for same day experiments.

Once in the laboratory, the connective tissue surrounding the adrenal gland was removed, and the clean adrenal gland was sliced into 1 mm thick slices using a tissue-slicer. The ZF and ZR layers were then manually dissected out of the 1 mm thick adrenal slices in the form of thin strips with the aid of scalpel blades, and were then cut into smaller fragments [14, 15]. The average size of a single ZF or ZR tissue fragment was 1 mm × 2 mm × 2 mm. The entire dissection procedure outlined above was conducted in RPMI medium at room temperature. After

the ZF and ZR tissue were isolated, 0.4 g of adrenal ZF or ZR tissue fragments were weighed out for each treatment sample. Each weighed tissue sample was incubated in a 25 ml Erlenmeyer flask containing 4 ml of RPMI medium. The flasks containing the tissue were incubated at 37°C in a rocking water bath (45 cycles per minute), and the flasks capped and aerated with 5% CO_2 **:** 95% O_2 using needle inserts. The ZF or ZR tissue fragments were exposed to the atmosphere of 5% CO_2 **:** 95% O_2 to maintain the proper pH and to prevent AMP-activated protein kinase (AMPK) activation due to tissue hypoxia.

Treatment of Adrenal Tissue Fragments

Each tissue sample was pre-equilibrated with the atmosphere of 5% CO_2 **:** 95% O_2 at 37°C for 30 minutes immediately before the start of the treatment experiments. After the fragments were pre-equilibrated, the RPMI medium was removed, the respective treatment mediums were added, and the experiments were performed as described below.

Immediately after the respective treatments were performed, the RPMI treatment medium was removed; the tissue samples were wrapped in aluminum foil, and flash frozen in liquid nitrogen. The flash frozen adrenal ZF or ZR tissue samples were then stored at -80°C until protein extractions or mRNA extractions were performed.

Time-Course Experiments

<u>IL-6 time-course</u>

Recombinant murine IL-6 (PeproTech, Rocky Hill, NJ, USA) was dissolved in RPMI medium and the stock solution was stored at -80°C until utilized in experiments. IL-6 was diluted to the desired concentration with RPMI medium immediately before the start of each experiment. For the time-course experiments, each adrenal ZF or ZR tissue sample was

incubated in 4 ml of RPMI medium containing 25 ng/ml recombinant murine IL-6 for 15, 30, 60, or 120 minutes. The control tissue samples were incubated in 4 ml of RPMI medium (without IL-6) under the same conditions and for the same time periods as the IL-6 treated counterparts.

AICAR time-course

Each adrenal ZF or ZR tissue sample was incubated in 4 ml of RPMI medium containing 1 mM AICAR (Santa Cruz Biotechnology, Dallas, TX, USA) for 30, 60, or 180 minutes. The control tissue samples were incubated in 4 ml of RPMI medium (without AICAR) under the same conditions and for the same time periods as the AICAR treated counterparts.

Dose-Response Experiments

IL-6 dose-response

Each adrenal ZF or ZR tissue sample was incubated for 60 minutes in 4 ml RPMI medium containing different concentrations of recombinant murine IL-6. The treatment concentrations were 0 ng/ml IL-6 (control-RPMI medium alone), 0.025, 0.1, 0.25, 1.0, 2.5, 10, or 25 ng/ml IL-6. In selected experiments, recombinant bovine IL-6 (Genway Biotech, San Diego, CA, USA) was utilized instead of the murine IL-6. Control tissue samples were incubated in 4 ml of RPMI medium (without IL-6) for 60 minutes, and were treated under the same conditions as the treatment samples.

AICAR dose-response

Each adrenal ZF or ZR tissue sample was incubated for 60 minutes in 4 ml of RPMI medium containing different concentrations of AICAR. The treatment concentrations were 0 mM (Control-RPMI medium alone), 0.1, 0.3, 0.5, 1.0, or 3.0 mM AICAR. The control tissue samples

were incubated in RPMI medium (without AICAR), and were treated under the same conditions as the treatment samples.

Inhibitor Experiments

Compound C Experiments

Compound C, (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), also known as dorsomorphin (Cayman Chemical Company, Ann Arbor, MI, USA), was utilized to inhibit AMPK activation in the adrenal ZF or ZR tissues fragments [60, 61, 62]. Compound C was dissolved in DMSO and the stock solution was stored at -80°C. To ensure that AMPK was inhibited before the start of the experimental procedure, the ZF and ZR fragments were pre-incubated for 60 minutes with 10 μM compound C in RPMI or control medium (RPMI alone) before the start of the treatments. At the end of the pre-incubation period, the RPMI medium was removed and replaced with 4 ml of RPMI medium containing control medium (RPMI alone), 10 μM compound C, 1 mM AICAR without compound C, 25 ng/ml IL-6 without compound C, 1 mM AICAR with 10 μM compound C, or 25 ng/ml IL-6 with 10 μM compound C. The tissue fragments were then incubated for 60 minutes with the various treatments. In a second series of experiments, the experiments were performed as explained above with the tissues exposed to IL-6 at concentrations of 0.25, 2.5, and 25ng/ml IL-6 in the presence or absence of 10 μM compound C.

SR11302 Experiments

SR11302 is an AP-1 inhibition specific retinoid; a functional inhibitor of AP-1 [63, 64]. SR11302 (Santa Cruz Biotechnology, Dallas, TX, USA) was dissolved in DMSO to achieve a concentration of 1 μM, and the stock solution was stored at -80°C. The experiments using

SR11302 were performed as described above for compound C, except 1 μM SR11302 was utilized in the pre-incubation and incubation steps instead of compound C.

RNA Extraction and Quantification

TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), also known as TRI reagent, was used to extract RNA from flash frozen adrenal ZF or ZR tissue samples. The ZF or ZR tissue samples were placed in glass homogenizer tubes, and 1 ml of TRIzol reagent per 100 mg of tissue was added to each sample. The tissue was homogenized until the tissue fragments were completely pulverized. The homogenized tissue samples were incubated for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes by the TRIzol reagent, and then transferred into new centrifuge tubes. Chloroform (0.2 ml per 1 ml of TRIzol reagent) was then added, samples vortexed for 10 seconds, and incubated at room temperature for 5 minutes. The samples were then centrifuged at 10000×g for 15 minutes at 4°C.

Following centrifugation the mixture separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Since RNA remains exclusively in the upper aqueous phase, it was transferred carefully into new tubes. Isopropyl alcohol (0.5 ml per 1 ml of TRIzol reagent used for the initial homogenization) was added to precipitate the RNA. The samples were incubated on ice for 10 minutes, and then centrifuged at 10000×g for 10 minutes at 4°C. The supernatant was completely removed. The gel-like RNA pellet which formed on the sides and bottoms of the tubes was washed once with 1 ml of 75% ethanol per 1 ml of TRIzol reagent used for the initial homogenization. The RNA pellets were then air-dried for 10 minutes. The extracted RNA pellets were then dissolved in 50 μl of nuclease free water and heated for 15 minutes in a 60°C water bath until the pellet completely dissolved.

A ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the RNA concentration of each RNA sample. Two µl of each RNA extract sample was placed on the nano-drop pedestal, and the ND1000-V3.5.1 program was used to measure the RNA concentrations. The RNA extracts were stored at -80°C until assayed.

Reverse Transcription of the RNA

The RNA samples of the ZF or ZR tissues which were extracted as described above were then reverse transcribed to cDNA. A grand mixture of reagents containing 2 µl 10× buffer, 1 µl dNTP (deoxynucleotide triphosphates), 1 µl RNase-out, 2 µl DTT, 0.4 µl random decamers, and 1 µl superscript-II-reverse-transcriptase was prepared. Each of the above amounts of reagents was multiplied by the number of ZF or ZR treatment samples. A 7.4 µl aliquot of the above grand mixture was then added into 2 µg of each RNA sample. The volume of RNA sample containing 2 µg of RNA was calculated in the quantification procedure described above. The total sample volume was then adjusted to 20 µl by the addition of nuclease free water. The samples were then centrifuged, vortexed, and loaded into a programmable thermocycler (Gene Amp PCR system 2400 Perkin Elmer, Waltham, MA, USA). The mixture samples were heated to 42°C for 60 minutes, followed by rapid cooling to 4°C in the programmable thermocycler. The reverse transcribed cDNA samples were then quantified by standard PCR using the primers listed in Table 1.

Standard PCR

Standard PCR reactions were performed on reverse transcribed cDNA samples of the ZF or ZR tissue. A grand mixture was prepared using 39.975 µl of nuclease free water, 5 µl of 10×buffer, 0.625 µl of 0.4mM dNTP mixture (deoxynucleotide triphosphates), 2 µl of 0.1 nM/µl mixture of sense and anti-sense of the respective primers, and 0.4 µl of Jstaq (250 units of DNA

polymerase). The above reagent volumes were multiplied by the number of treatment samples, and separate grand mixtures were prepared for the different primers used. The primers used for the standard PCR were designed using primer design software from Invitrogen, Vector NTI, and AB1, Primer Express. Primers were designed for StAR, DAX-1, SF-1, P450scc, and 18s ribosomal RNA (Table 1). Two µl of each RT (reverse transcribed cDNA) was added to 48 µl of the above grand mixture to achieve a total volume of 50 µl in each sample. The PCR tubes were then lightly vortexed, centrifuged and loaded into the programmable thermocycler.

Initially the thermocycler was programmed to 95º C for 3 minutes, which denatured the cDNA. Following the initial denaturing step the thermocycler was set to 35 to 55 cycles of temperatures. The first temperature was programmed to 95º C for 20 to 30 seconds, depending on the length of the amplified sequence. This step denatured the template and newly made complementary strand. The second temperature was programmed for 20 to 30 seconds to temperatures ranging from 55ºC to 58º C depending on the length of the primer and the percentage of purines and pyrimidines. This step annealed the primer to the target cDNA. The third temperature was programmed to 72º C for 20 to 60 seconds, depending on the length of the amplified sequence. This step provided the optimal temperature for polymerase function and elongation of the amplified sequence. The process ended with a final temperature of 72º C for 3 minutes for the final elongation, and 4º C for 5 minutes for stopping further reactions. The DNA samples were then frozen at -20º C until analyzed.

The PCR products were analyzed by gel electrophoresis. PCR samples were mixed with 2 µl of loading dye (BioRAD, Hercules, CA, USA), and electrophoresed in a 0.5 % agarose gel containing ethidium bromide. The DNA samples were electrophoresed at 80V for 30 minutes. The gel was then visualized under ultraviolet illumination using a Fluorochem 8900 Imaging

system (Alpha Innotech Corporation, Santa Clara, CA, USA). Quantification of the cDNA bands was then performed using the SpotDensity tool of the AlphaEase Fluorochem 8900 software (Alpha Innotech Corporation, Santa Clara, CA, USA).

Table 1: Nucleotide sequences of the primers used for standard PCR

Gene amplified	Nucleotide sequence of primer	Primer type	Number of base pairs
StAR	5'-CCT CTC TAC AGC GAC CAA-3'	Sense	311
	5'-TCG TGA GTG ATG ACC GTG-3'	Anti-sense	
P450scc	5'-AAC GTC CCT CCA GAA CTG TAC C-3'	Sense	358
	5'-CTT GCT TAT GTC TCC CTC TGC C-3'	Anti-sense	
SF-1	5'-AGT TTG GGC CCA TGT ACA AGC G-3'	Sense	290
	5'-GCA GGG TAG AGG TAG CCA GCC A-3'	Anti-sense	
DAX-1	5'-AGG GGA CCG TAC TCT TCA AC-3'	Sense	214
	5'-ATG ATG GGC CTG AAG AAC AG-3'	Anti-sense	
18s	5' –GTAACCCGTTGAACCCCATT-3'	Sense	20
	5' –CCATCCAATCGGTAGTAGCG-3'	Anti-sense	

Protein Extraction and Quantification

Proteins were extracted from the ZF or ZR tissue samples using protein extraction buffer containing 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, and 10 ml glycerol per 100 ml distilled water, at a pH of 7.4. Immediately before performing the extraction, dithiothreitol (1 mM), benzamidine (1 mM), soybean trypsin inhibitor (5 μg/ml), and phenylmethanesulfonylfluoride (0.1 mM) were added to the above described protein extraction buffer. Four ml of extraction buffer mixture per 1 g of ZF or ZR tissue were added to the samples in glass homogenizer tubes; homogenized completely, and then incubated on ice for 60 minutes. The samples were centrifuged at 10000×g for 10 minutes at 4°C, and the supernatants were transferred to new tubes. A micro-Bradford assay was performed using a ND-1000 spectrophotometer (NanoDrop Technologies,

Wilmington, DE, USA) to determine the protein concentrations of each sample. The protein extracts were stored at -80°C until assayed.

Western Blot

Western blots were performed on the protein extracts of the treated ZF or ZR tissue in order to investigate the effects of the different treatments on expression of steroidogenic proteins. The protein samples for SDS –PAGE electrophoresis were prepared by adding a mixture of SDS-PAGE sample buffer loading dye (BioRAD, Hercules, CA, USA), β-mercaptoethanol (BME), the respective volumes of ZF or ZR protein extract (80 µg) according to the performed micro-Bradford assay, and nuclease free water. The prepared protein samples were loaded into 12% Tris-HCl-Ready-Gels (BioRad, Hercules, CA, USA). A western blot protein standard ladder (BioRad, Hercules, CA, USA) was loaded into the first well of each gel to estimate the molecular weight of the protein bands. The electrophoresis was performed at 200 V for 30 minutes. The proteins on the gels were then transferred to Immunoblot PVDF membrane (BioRAD, Hercules, CA, USA) using transfer apparatus at 100 V for 60 minutes. The PVDF membranes were stained with Ponceau S staining solution (Sigma-Aldrich, St. Louis, MO, USA) to test for equal loading. The Ponceau S stain was later washed off with 1×TBST for 5 minutes.

Next, the PVDF membranes were incubated in blocking solution (5% non-fat dry-milk in 1×TBST) for 60 minutes on a rocker at room temperature in order to block non-specific binding. The membranes were then washed in 1×TBST for 5 minutes. The PVDF membranes were incubated protein-side-up in the respective primary antibody diluted in1×TBST, overnight at 4ºC. Following overnight incubation, the membranes were washed in 1×TBST for 15 minutes, and then incubated in the respective secondary antibody for 60 minutes on a rocker at room temperature. At the end of the secondary antibody incubation the membranes were washed in

1×TBST for 15 minutes. The PVDF membranes were then incubated in western blotting Luminol reagent (Santa Cruz Biotechnology, Dallas, TX, USA) for 2 minutes. The PVDF membranes were then exposed to X-ray film in a dark-room, and the X-ray film was developed in a X-ray developer. The developed X-ray film was then scanned and analyzed using a Fluorochem 8900 Imaging system (Alpha Innotech Corporation, Santa Clara, CA, USA). Quantification of the protein bands was then performed using the SpotDensity tool of the AlphaEase Fluorochem 8900 software (Alpha Innotech Corporation, Santa Clara, CA, USA).

The primary antibodies used were rabbit anti-human StAR (sc-25806, 1:500); goat anti - human P450scc (sc-18043, 1:2000); rabbit anti-human SF-1 (sc-28740, 1:800); and rabbit anti-human DAX-1 (sc-841, 1:500); rabbit anti-human cFOS (sc-7202, 1:800); rabbit anti-human cJUN (sc-1694, 1:1000); rabbit anti-human JUNB (sc-37, 1:600); JUND (sc-74, 1:800); rabbit anti-human phospho-cJUN (sc-101721, 1:900) obtained from Santa Cruz Biotechnology, Dallas, TX, USA. The primary antibodies rabbit-pACC (catalog no. 3661, 1:3000) and rabbit-pAMPKα (catalog no. 4188, 1:1000) were obtained from Cell Signaling, Boston, MA, USA. The secondary polyclonal antibodies used were goat anti-rabbit IgG complexed to horse radish peroxidase (sc-2030, 1:500) and donkey anti-goat IgG complexed to horse radish peroxidase (sc-2033, 1:500) was obtained from Santa Cruz Biotechnology, Dallas, TX, USA.

Statistical Analysis

All experimental procedures were repeated at least three times. The data from the standard PCR were quantified by dividing the absorbance of each band of the treatment group on a gel by the absorbance of the corresponding 18S bands on the same gel. This ratio was then normalized by dividing it by the control treatment band/18S ratio of the control sample at 60 minutes on the same gel. The data from the western blots were normalized by dividing the

relative density of the resulting band by the control value at 60 minutes. Statistical analysis was performed by one way analysis of variance (ANOVA) and the Bonferroni post test for multiple comparisons utilizing NCSS software (NCSS Statistical Software, Kaysville, UT, USA). The data were analyzed to determine statistical significance at both $P < 0.05$ and $P < 0.01$. The data were graphed utilizing SigmaPlot software (Academic Distributing Inc., Chicago, IL, USA). The points or bars in each figure represent the mean plus or minus the standard error of the mean (S.E.M.). The significance levels ($P < 0.05$ or $P < 0.01$) are indicated by alphabetic letters above each point or bar, and the number of samples at each point is indicated by the numerals above or below the error bars.

CHAPTER 3: INTERLEUKIN-6 INCREASES THE EXPRESSION OF STAR AND P450SCC IN THE ADRENAL ZONA FASCICULATA THROUGH ACTIVATION OF AMPK-ACTIVATED PROTEIN KINASE

Introduction

Cortisol release from the adrenal zona fasciculata (ZF) is thought to be primarily regulated by adrenocorticotrophic hormone (ACTH) from the anterior pituitary [1, 2]. Although there is a good correlation between ACTH and cortisol release during periods of acute stress, during chronic inflammatory stress ACTH returns to normal physiological concentrations whereas cortisol in humans and corticosterone in rodents remain elevated [1, 2, 3, 6, 7]. Therefore it has been hypothesized that various cytokines including interleukin-6 (IL-6) may play a role in the regulation of cortisol and corticosterone release from the ZF during chronic inflammatory stress. Inflammation increases the plasma concentrations of IL-6, and various experiments support a role for IL-6 in regulating cortisol or corticosterone release during chronic inflammatory stress [1, 2, 3, 6, 7, 8, 10]. Although IL-6 increases cortisol release from dispersed bovine adrenal ZF cells [14, 16], the biochemical mechanisms involved in this release of cortisol have not yet been determined.

Previous research performed in our laboratory has shown that IL-6 increases the expression of steroidogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (P450scc) in the ZF tissue [16]. These two steroidogenic proteins regulate rate-limiting steps in steroidogenesis [26, 27, 28, 30]. StAR regulates the transport of cholesterol into the mitochondrion [26, 27, 28], and P450scc cleaves cholesterol into pregnenolone [30]. IL-6 also increases the expression of steroidogenic factor 1 (SF-1) in the adrenal ZF; a nuclear factor that enhances the expression of steroidogenic proteins [14, 16]. Meanwhile IL-6 decreases the

expression of dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-1) in the ZF [14, 16]. Thus, the IL-6- induced increase in SF-1 and decrease in DAX-1 may mediate the increase in StAR and P450scc [65, 66, 67]. However, the mechanism through which IL-6 modifies SF-1 and DAX-1 expression has not been determined.

AMP-activated protein kinase (AMPK) plays a key role in the regulation of metabolism in many tissues [51, 52, 53]. In addition to its vital roles in regulating cell metabolism, AMPK has been shown to play a role in the regulation of steroidogenesis in endocrine tissues such as the testes, ovary and adrenal gland [16, 47, 48, 49, 50]. Recent studies have shown that AMPK is present and is activated in the adrenal cortex in response to chronic inflammatory stress [50]. The peptide adiponectin increases cortisol release from H295R cells, and this increase is accompanied by an activation of AMPK as indicated by AMPK phosphorylation [47]. Inhibition of AMPK activity blocked the adiponectin-induced increase in cortisol release from the H295R cells [47]. Except for these studies, the role of AMPK in steroidogenesis in the adrenal cortex is a fairly unstudied topic of research. Since AMPK mediates the effects of IL-6 in other tissues [48, 51, 52], the role of AMPK in mediating the effects of IL-6 on adrenal ZF steroidogenesis was examined. It was hypothesized that IL-6 activates AMPK in the ZF, which would then lead to increased expression of StAR and P450scc.

Results

AICAR Effects on AMPK Activation in the ZF

The AMPK activator AICAR was tested for its ability to activate AMPK in the adrenal ZF tissue fragments. AICAR dose-response and time-course experiments were performed as described in Materials and Methods, and protein levels of PACC and PAMPK in response to

AICAR stimulation were measured by western blots. Phosphorylation of AMPK is a key indicator of AMPK activation [68]; thereby PAMPK levels are a measure of AMPK activation. As shown in Fig. 3-1, the protein expression level of PAMPK increased in both the dose-response (Fig. 3-1-C) and time-course (Fig. 3-1-D) experiments. Further downstream, the activated AMPK activates acetyl-CoA-carboxylase (ACC) by phosphorylation [68]. Thus PACC is an index of the activity of AMPK. As shown in Fig. 3-1, the protein expression level of PACC also increased in both dose response (Fig. 3-1-A) and time-course (Fig. 3-1-B) experiments. Thus, these results demonstrate both a dose-dependent and time-dependent increase in the levels of PACC and PAMPK in response to AICAR exposure. Therefore, AICAR activated AMPK in the adrenal ZF tissue.

IL-6 Effects on AMPK Activation in the ZF

The effects of IL-6 on AMPK activation in adrenal ZF tissue fragments were studied in order to determine if IL-6 regulates the expression of adrenal steroidogenic proteins through AMPK activation. IL-6 dose-response and time-course experiments were performed as described in Materials and Methods, and the protein levels of PACC and PAMPK in response to IL-6 stimulation were measured using western blots. The protein expression level of PAMPK increased in both the dose-response (Fig-3-2-C) and time-course (Fig. 3-2-D) experiments in response to IL-6 treatment. Furthermore, the protein expression level of PACC increased in both dose-response (Fig. 3-2-A) and time-course (Fig. 3-2-B) experiments in response to IL-6 treatment. Accordingly the results indicate that IL-6 increased AMPK activation in the adrenal ZF tissue fragments.

Effects of AMPK Activation on Steroidogenic Factors in the ZF

Since the above experiments demonstrated that AICAR stimulates AMPK activation in the adrenal ZF tissue, the effects of AMPK activation on the adrenal steroidogenic factors StAR, P450scc, DAX-1 and SF-1 in the ZF were determined. AICAR dose-response and time-course experiments were performed as described in Materials and Methods to assess the effects of increasing AMPK activation in the ZF tissue fragments. Protein levels of StAR, P450scc, DAX-1 and SF-1 were measured using western blots; and mRNA levels of StAR, P450scc, DAX-1 and SF-1 in response to AICAR stimulation were measured using standard RT-PCR as described in Materials and Methods.

StAR protein transports cholesterol into the mitochondrion and controls the rate limiting step in adrenal steroidogenesis [26, 27, 28]. Thus, the mRNA and protein expression levels of StAR are a predictor of the rate of adrenal steroidogenesis. The mRNA expression level of StAR increased in both the dose-response (Fig. 3-3-A) and time-course (Fig. 3-3-C) experiments in response to AICAR treatment. Likewise, the protein expression level of StAR increased in both the dose-response (Fig. 3-3-B) and time-course (Fig. 3-3-D) experiments in response to AICAR treatment in the ZF tissue fragments.

P450scc also serves as a measure of adrenal steroidogenesis since P450scc acts as the enzyme which cleaves cholesterol into pregnenolone [30]. The mRNA expression level of P450scc increased in both the dose-response (Fig. 3-4-A) and time-course (Fig. 3-4-C) experiments in response to AICAR treatment. Also, the protein expression level of P450scc increased in both the dose-response (Fig. 3-4-B) and time-course (Fig. 3-4-D) experiments in response to AICAR treatment in the ZF tissue fragments.

Steroidogenic factor-1 (SF-1) is a transcription factor that up regulates adrenal steroidogenesis in the ZF, and is therefore a predictor of the adrenal steroidogenic equilibrium in the ZF tissue [31, 32, 34, 35]. The mRNA expression level of SF-1 increased in both the dose-response (Fig. 3-5-A) and time-course (Fig. 3-5-C) experiments in response to AICAR treatment. Similarly, the protein expression level of SF-1 also increased in both the dose-response (Fig. 3-5-B) and time-course (Fig. 3-5-D) experiments in response to AICAR treatment of the ZF tissue fragments.

DAX-1 is a transcription factor that down-regulates adrenal steroidogenesis, and can therefore be used as a measure of the direction of adrenal steroidogenesis. Higher levels of DAX-1 indicate increased inhibition of adrenal ZF steroidogenesis, whereas lower levels of DAX-1 indicate stimulation of adrenal ZF steroidogenesis [37, 38, 39, 40, 41, 42, 43]. The mRNA expression level of DAX-1 decreased in both the dose-response (Fig. 3-6-A) and time-course (Fig. 3-6-C) experiments in response to AICAR treatment. Likewise, the protein expression level of DAX-1 decreased in both the dose-response (Fig. 3-6-B) and time-course (Fig. 3-6-D) experiments in response to AICAR treatment in the ZF tissue fragments.

Overall, increasing AMPK activation through increased concentrations of AICAR and longer incubation times in AICAR led to an increase in StAR, P450scc, SF-1, and a decrease in DAX-1 in the adrenal ZF tissue fragments. As StAR, P450scc, and SF-1 are stimulatory factors, and DAX-1 is an inhibitory factor, AMPK activation can be concluded to favor steroidogenesis in the ZF, thus suggesting that AMPK activation may lead to increased cortisol synthesis in the ZF.

Effects of AMPK Inhibition on Steroidogenic Factors in the ZF

Since the previous experiments provided evidence that AMPK activation by AICAR and IL-6 increases StAR, P450scc, SF-1, and decreases DAX-1, the role of AMPK in modifying these proteins was studied utilizing an inhibitor of AMPK. The AMPK inhibitor, compound C, was used to inhibit AMPK in both the presence of IL-6 and AICAR. Adrenal ZF tissue fragments were treated with compound C alone, IL-6 alone, and AICAR alone, and also with combinations of IL-6 + compound C, and AICAR + compound C. The ZF tissue protein extracts were then western blotted for PACC, PAMPK, StAR, P450scc, SF-1, and DAX-1.

In order to confirm that compound C inhibits AMPK activation in ZF tissue, western blots were conducted for PACC and PAMPK in ZF tissue treated with compound C. Compound C, in the presence of IL-6 and AICAR, significantly reduced the levels of PACC (Fig. 3-7-E) and PAMPK (Fig. 3-7-F) as compared to IL-6 and AICAR alone. These results indicate that compound C acts as a blocker of AMPK activation in the ZF tissue fragments. Compound C also inhibited the AICAR and IL-6 stimulation of StAR (Fig. 3-7-A), P450scc (Fig. 3-7-B), and SF-1 (Fig. 3-7-C). Compound C prevented AICAR and IL-6 from inhibiting DAX-1 (Fig. 3-7-D) as compared to AICAR and IL-6 alone.

The effect of compound C on different concentrations of IL-6 was assessed in order to determine whether compound C blocked the stimulatory effects of IL-6 on StAR at all IL-6 concentrations, or if the compound C inhibition was limited to only 25 ng/ml IL-6 concentration. Adrenal ZF tissue was exposed to IL-6 at 0.25, 2.5, or 25 ng/ml concentrations in the presence or absence of 10 μM compound C, and the StAR protein expression was determined by western blots. StAR protein expression increased with increasing concentrations of IL-6, and the increase

in StAR expression by IL-6 was reversed by compound C at all three concentrations of IL-6 (Fig. 3-8).

Discussion

The data presented in this study support a role for AMPK in the regulation of steroidogenic proteins in the adrenal ZF, and a specific role for AMPK in IL-6 mediated modification of these steroidogenic proteins. AMPK activation by AICAR in the ZF increases the expression of the steroidogenic enzymes StAR (Fig. 3-3) and P450scc (Fig. 3-4) in a time-dependent and dose-dependent manner. AMPK activation also increases the expression of SF-1 (Fig. 3-5), and decreases the expression of DAX-1 protein and mRNA in a time-dependent and dose-dependent manner (Fig. 3-6). These results support the hypothesis that the AICAR-induced changes in StAR, P450scc, SF-1, and DAX-1 are mediated by AMPK activation in that ZF tissue exposed to AICAR has increased phosphorylation of AMPK indicating AMPK activation (Figs. 3-1-C-D). Furthermore, AICAR increases the phosphorylation of ACC in the ZF tissue, and ACC phosphorylation is an index of AMPK activation (Figs. 3-1-A-B). These results of AICAR effects on AMPK and ACC phosphorylation in the ZF confirm previous observations in H295R cells [50]. Moreover, the AMPK inhibitor compound C blocks the effects of AICAR on StAR, P450scc, SF-1, and DAX-1 protein expression in the adrenal ZF tissue demonstrating that these effects of AICAR are probably mediated through AMPK activation (Figs. 3-7-A-D). Furthermore, the compound C inhibition of AICAR phosphorylation of AMPK and ACC provides evidence that compound C is an inhibitor of AMPK activation in the ZF tissue (Figs. 3-7-E-F).

The hypothesis that AMPK is involved in the IL-6 regulation of the expression of steroidogenic enzymes in the adrenal ZF is supported by the observation that IL-6 activates

AMPK in the ZF of the adrenal gland as indicated by a dose-dependent and time-dependent increase in the levels of PAMPK (Figs. 3-2-C-D) and PACC (Figs. 3-2-A-B) in response to IL-6 exposure. Furthermore, as shown in previous studies, IL-6 affects StAR, P450scc, SF-1 and DAX-1 expression in the ZF similarly to AICAR. IL-6 increases the expression of StAR, P450scc, SF-1, and decreases the expression of DAX-1 [14, 16]. Compound C blocks the effects of IL-6 on the expression of StAR, P450scc, SF-1 and DAX-1 (Figs. 3-7-A-D), providing further evidence that IL-6 is functioning through AMPK activation. Furthermore, compound C inhibits the actions of IL-6 on StAR expression at IL-6 concentrations ranging from 0.25 ng/ml to 25 ng/ml of IL-6, indicating that AMPK activation is mediating an increase in StAR expression over a wide range of IL-6 concentrations (Figs. 3-8).

Furthermore, compound C had no effect on the increase in expression of steroidogenic proteins mediated by exposure of the ZF tissue to ACTH (data not presented). ACTH-stimulation of cortisol release is primarily mediated by an increase in intracellular cAMP [69]. The possibility that compound C is having a non-specific toxic effect upon the ZF tissue is refuted by the observation that the ACTH-stimulated increase in steroidogenic proteins is not affected by compound C (data not presented). Additionally, the increase in DAX-1 in response to compound C also indicates that compound C is not toxic on the ZF tissue fragments (Fig. 3-7-D).

According to our knowledge, the adrenal cortex is the second tissue after skeletal muscle in which IL-6 has been demonstrated to modify cellular function through an AMPK-dependent mechanism [70, 71, 72]. Although the majority of experiments with IL-6 presented in this study utilized recombinant murine IL-6, selected experiments were repeated with recombinant bovine IL-6 (data not presented), and the data from the recombinant bovine IL-6 were identical to the recombinant murine IL-6 data.

In general, these results suggest that AMPK activation is necessary for the IL-6-induced increased expression of StAR and P450scc in the adrenal ZF tissue. Similarly, in H295R cells AMPK activation appeared to be essential for adiponectin-induced increase in StAR expression and cortisol release [47]. It is not known if AMPK activation alone is sufficient for the activation of cortisol release from the ZF, and future experiments measuring cortisol release in cultured ZF cells will be used for this purpose. It is possible to hypothesize that the inhibition of IL-6-induced StAR and P450scc expression mediated by compound C will result in decreased cortisol release. However, proving this hypothesis will require additional experiments in which cortisol release is determined from cultured ZF cells in the presence and absence of IL-6 and compound C, because cultured ZF cells release hormones more robustly than ZF tissue fragments. The mechanism through which IL-6 treatment and AMPK activation by AICAR increases StAR and P450scc expression may be directly mediated by these agents increasing the expression of the nuclear factor SF-1 and decreasing the expression of the nuclear factor DAX-1. However, it is also possible that other nuclear factors may also be involved in this regulation, as well as any other unidentified intermediates.

In conclusion, AMPK may be involved in the regulation of steroidogenesis in the ZF during conditions such as hypoxia which alters AMPK activation and cortisol release, and under chronic stress conditions. Thus AMPK activation may play a role in the increased adrenal cortisol release from the ZF during IL-6 mediated chronic inflammatory stress. The above findings shed light into the basis of how increased IL-6 levels during chronic inflammatory stress leads to increased levels of cortisol in pathological conditions, and could have potential implications in treating the complications associated with chronic inflammatory stress.

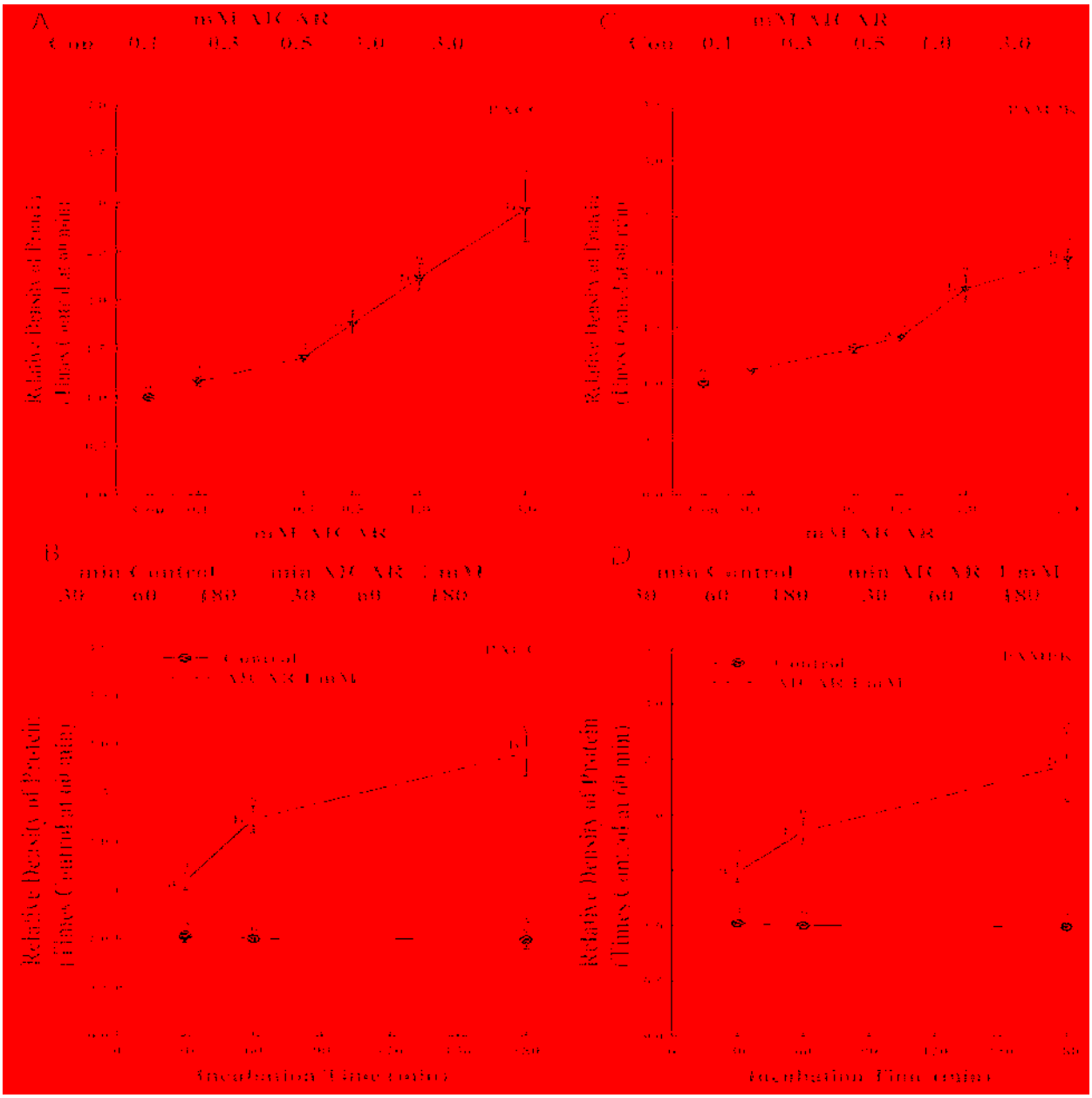

Figure 3-1: The dose-dependent and time-dependent effects of AICAR on the expression of PACC and PAMPK in the ZF

PACC (A, B) or PAMPK (C, D) protein expression was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment protein bands divided by the absorbance of the control treatment bands at 60 min. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiments (A, C):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.1, 0.3, 0.5, 1.0, or 3.0 mM AICAR. **Time course experiments (B, D):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 min. with RPMI medium (Control) or 1 mM AICAR.

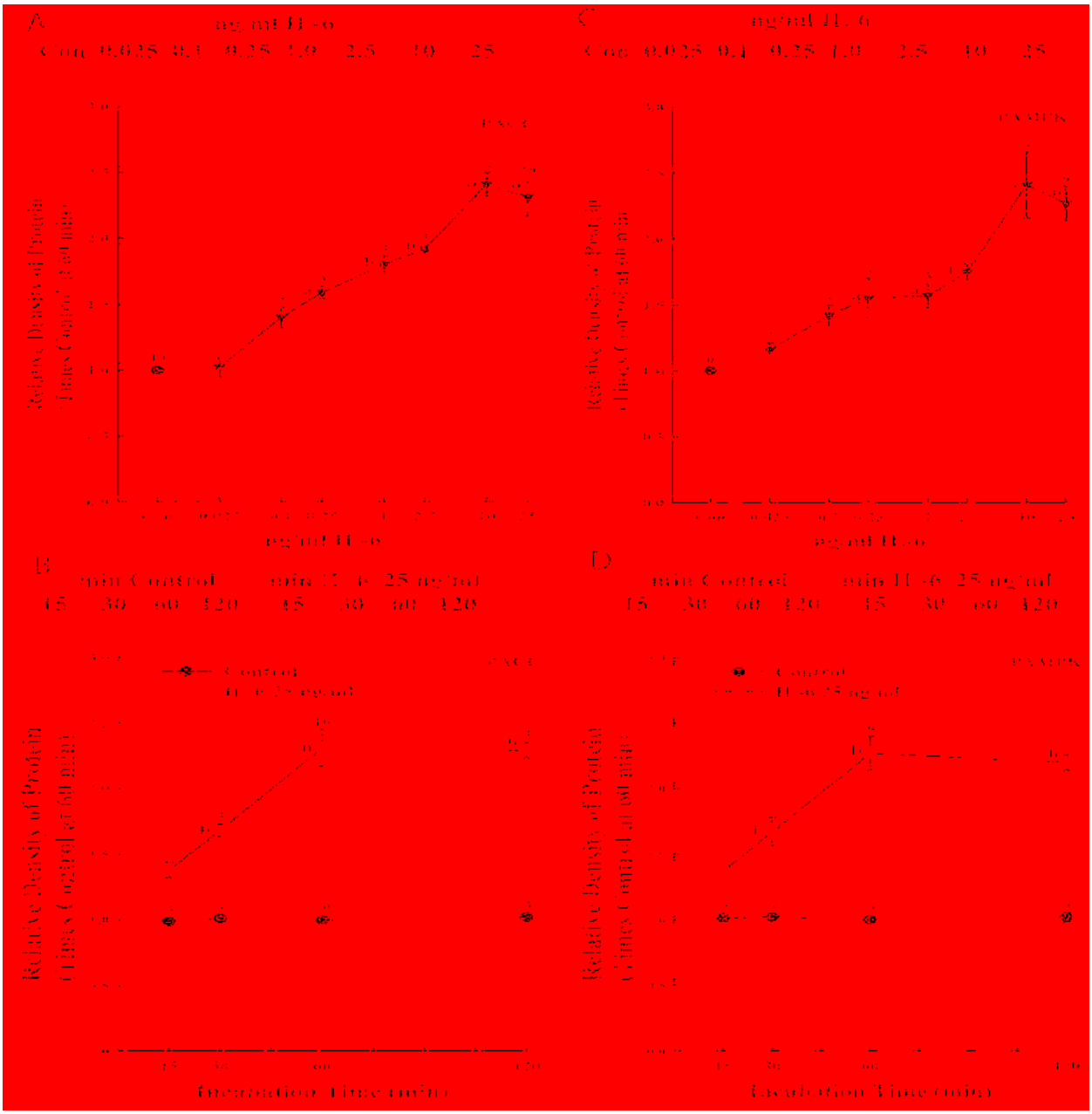

Figure 3-2: The dose-dependent and time-dependent effects of IL-6 on the expression of PACC and PAMPK in the ZF

PACC (A, B) or PAMPK (C, D) protein expression was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment protein bands divided by the absorbance of the control treatment bands at 60 min. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, C):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10, or 25 ng/mL IL-6. **Time-course experiments (B, D):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/mL IL-6.

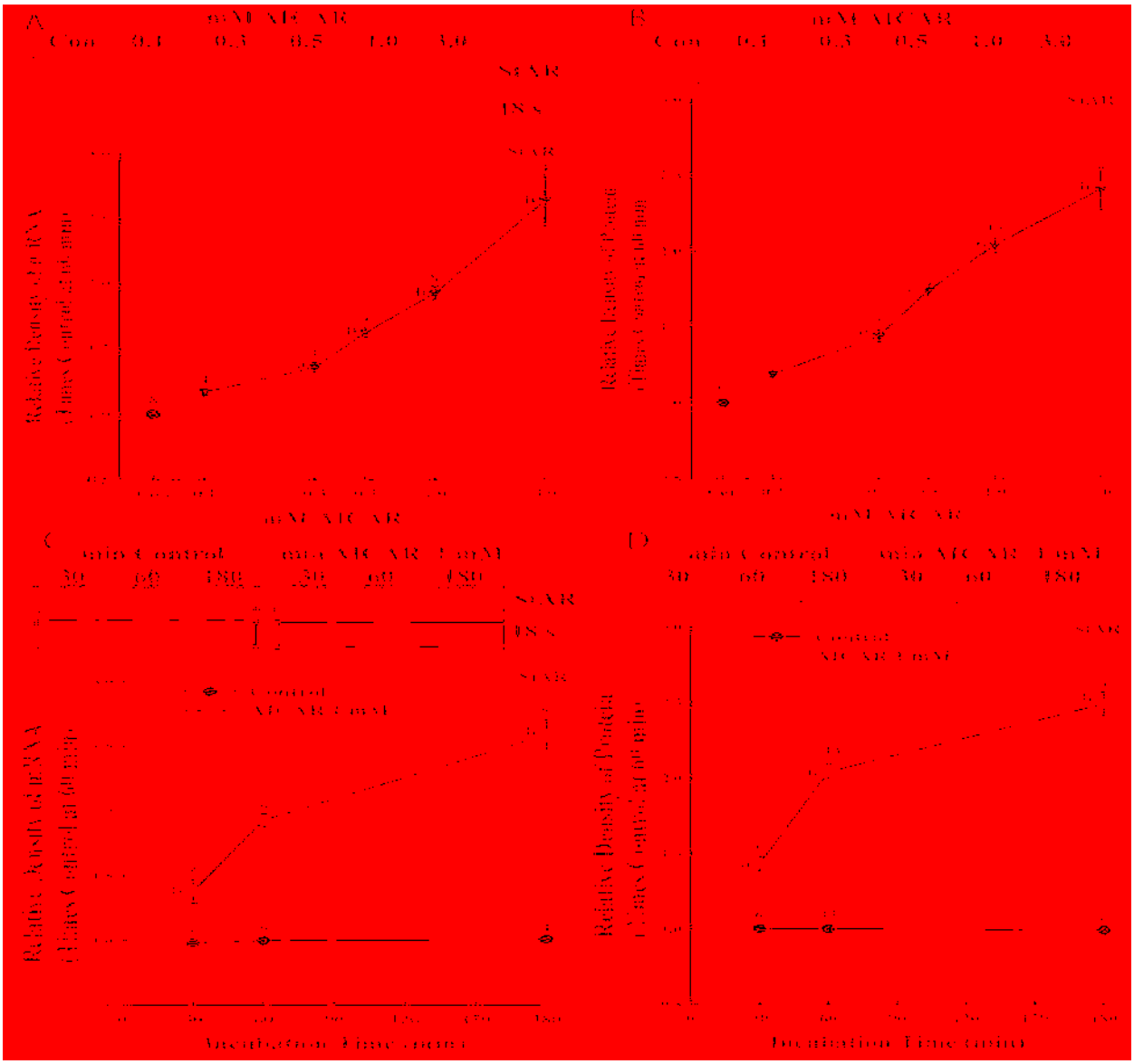

Figure 3-3: The dose-dependent and time-dependent effects of AICAR on the expression of StAR mRNA and protein in the ZF

StAR mRNA expression (A, C) was determined by RT-PCR and StAR protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each StAR band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the StAR mRNA/18s ratio of the control sample at 60 min on the same gel. The StAR protein expression was quantified by determining the ratio of the absorbance of the treatment StAR bands divided by the absorbance of the control treatment StAR bands at 60 min. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

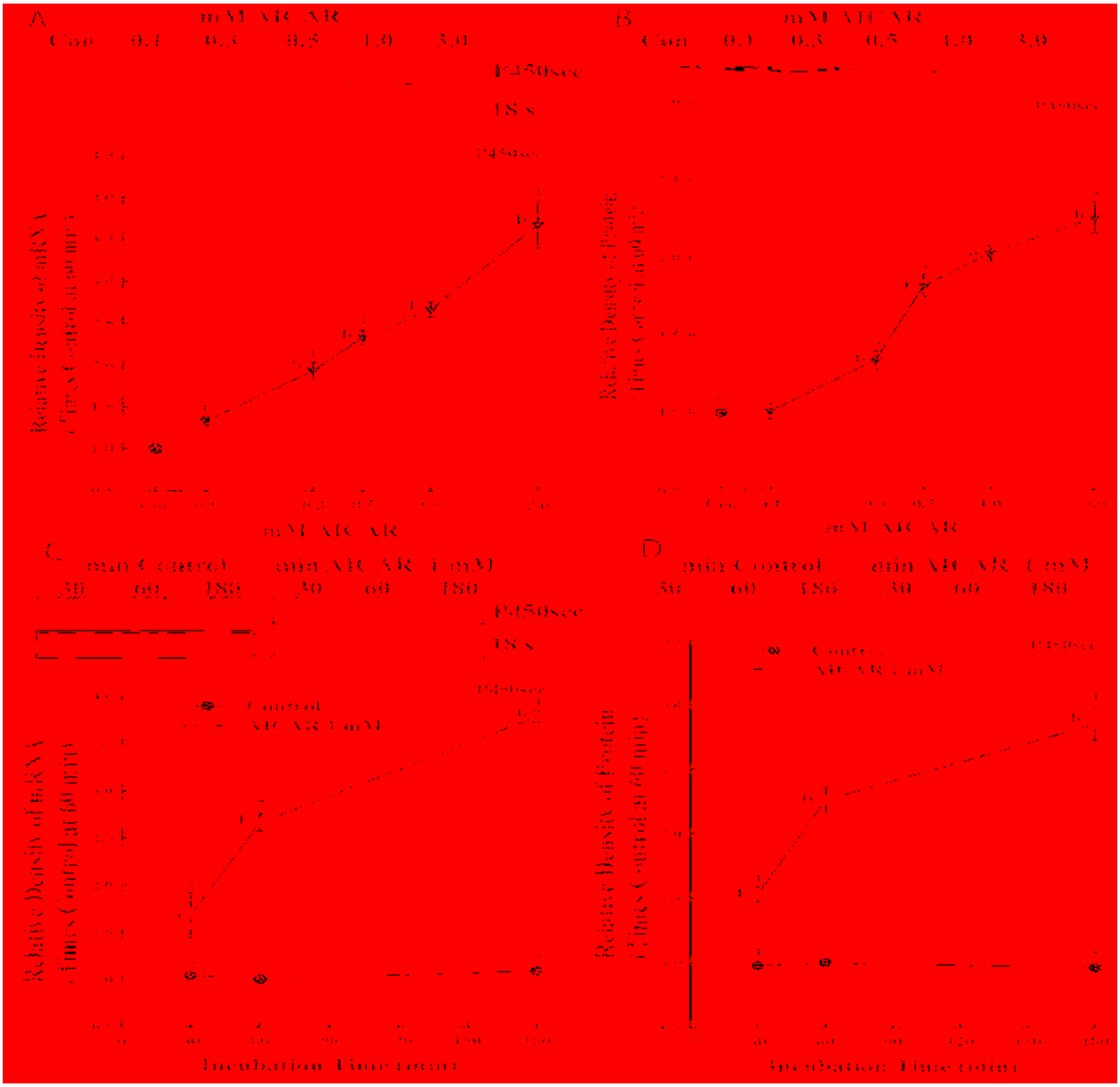

Figure 3-4: The dose-dependent and time-dependent effects of AICAR on the expression of P450scc mRNA and protein in the ZF

P450scc mRNA expression (A, C) was determined by RT-PCR and P450scc protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each P450scc band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the P450scc mRNA/18s ratio of the control sample at 60 minutes on the same gel. The P450scc protein expression was quantified by determining the ratio of the absorbance of the treatment P450scc bands, divided by the absorbance of the control treatment P450scc bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

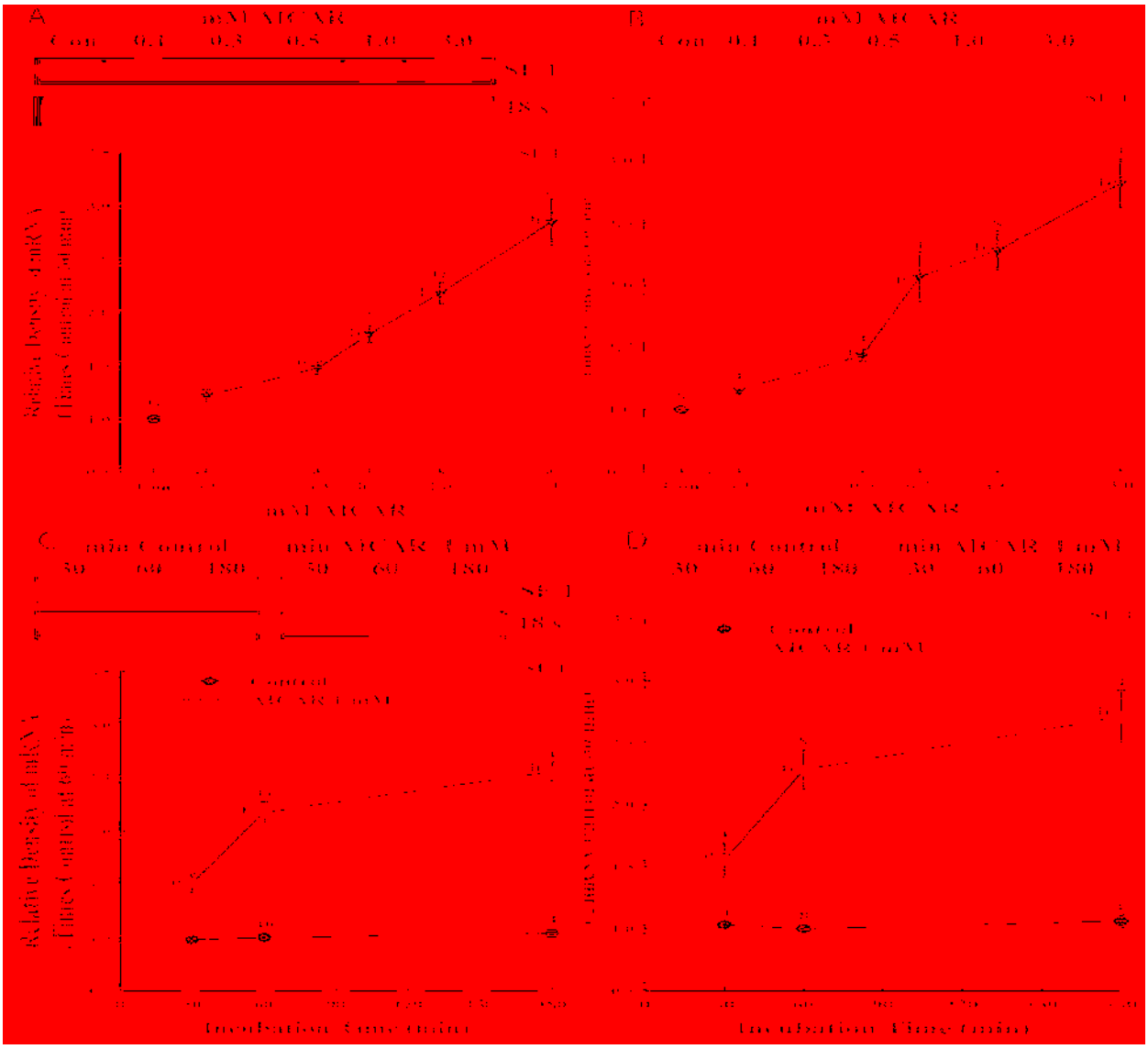

Figure 3-5: The dose-dependent and time-dependent effects of AICAR on the expression of SF-1 mRNA and protein in the ZF

SF-1 mRNA expression (A, C) was determined by RT-PCR, and SF-1 protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each SF-1 band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the SF-1 mRNA/18s ratio of the control sample at 60 minutes on the same gel. The SF-1 protein expression was quantified by determining the ratio of the absorbance of the treatment SF-1 bands divided by the absorbance of the control treatment SF-1 bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

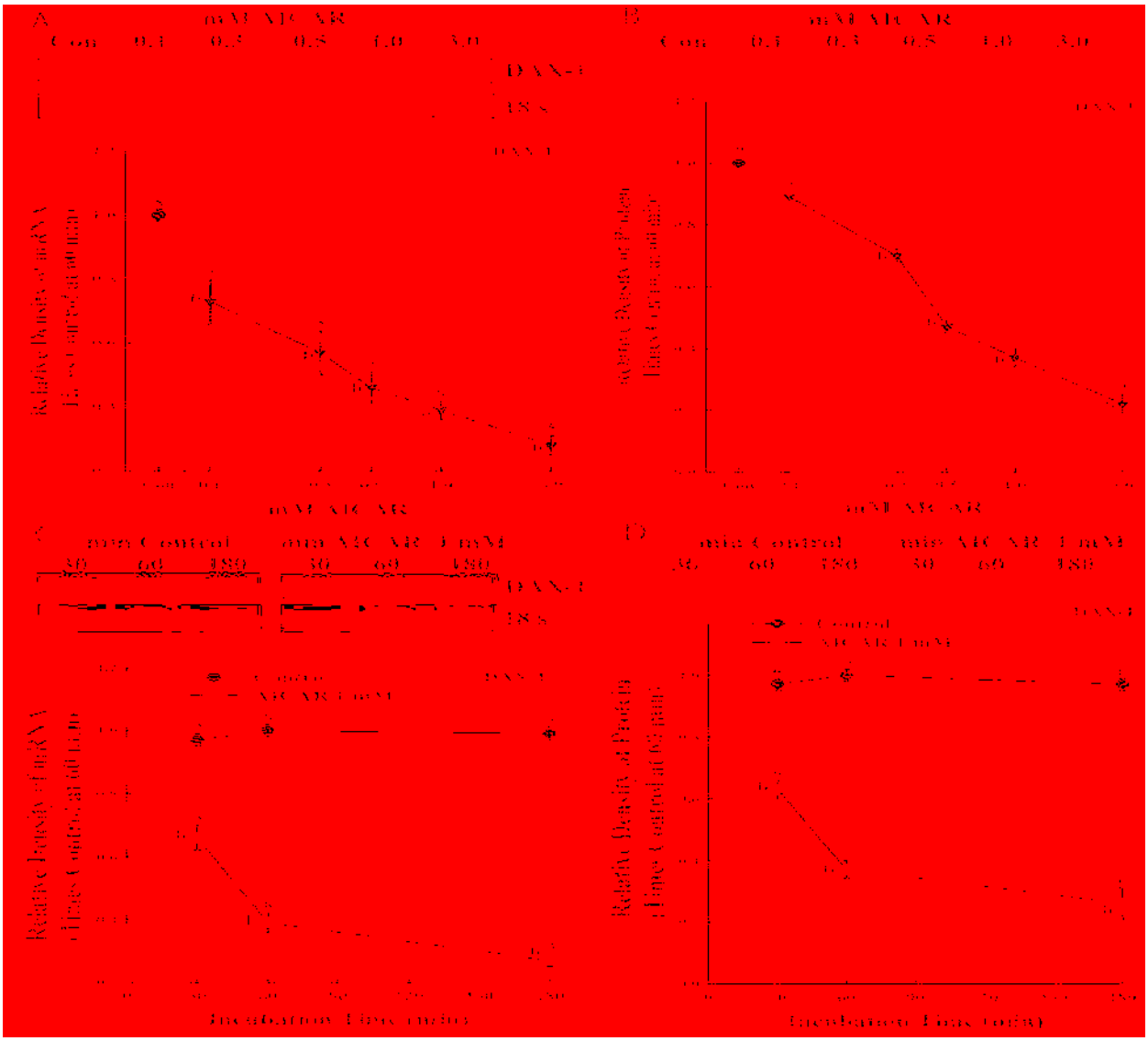

Figure 3-6: The dose-dependent and time-dependent effects of AICAR on the expression of DAX-1 mRNA and protein in the ZF

DAX-1 mRNA expression (A, C) was determined by RT-PCR, and DAX-1 protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each DAX-1 band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the DAX-1 mRNA/18s ratio of the control sample at 60 minutes on the same gel. The DAX-1 protein expression was quantified by determining the ratio of the absorbance of the treatment DAX-1 bands divided by the absorbance of the control treatment DAX-1 bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

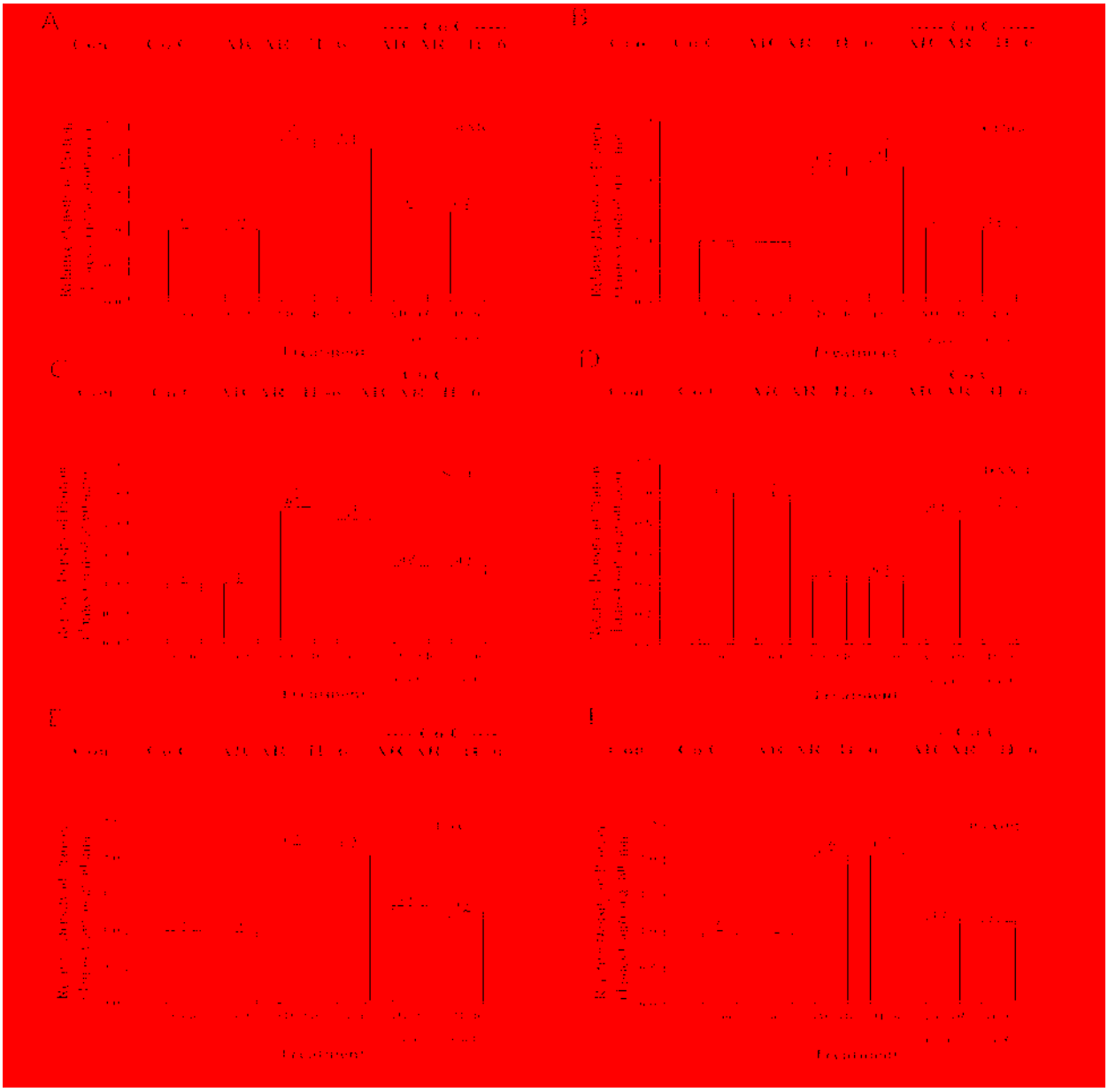

Figure 3-7: The effects of compound C on IL-6-stimulated and AICAR-stimulated expression of steroidogenic factors, PACC and PAMPK in the ZF

Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium or 10 µM compound C. The incubation medium was then removed and the ZF fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR + 10 µM compound C; 25 ng/ml IL-6 + 10 µM compound C. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated representative western blots for (A) StAR; (B) P450scc; (C) SF-1; (D) DAX-1); (E) PACC; (F) PAMPK.. The lower section of each panel is the combined results from multiple experiments with the bar representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6)).

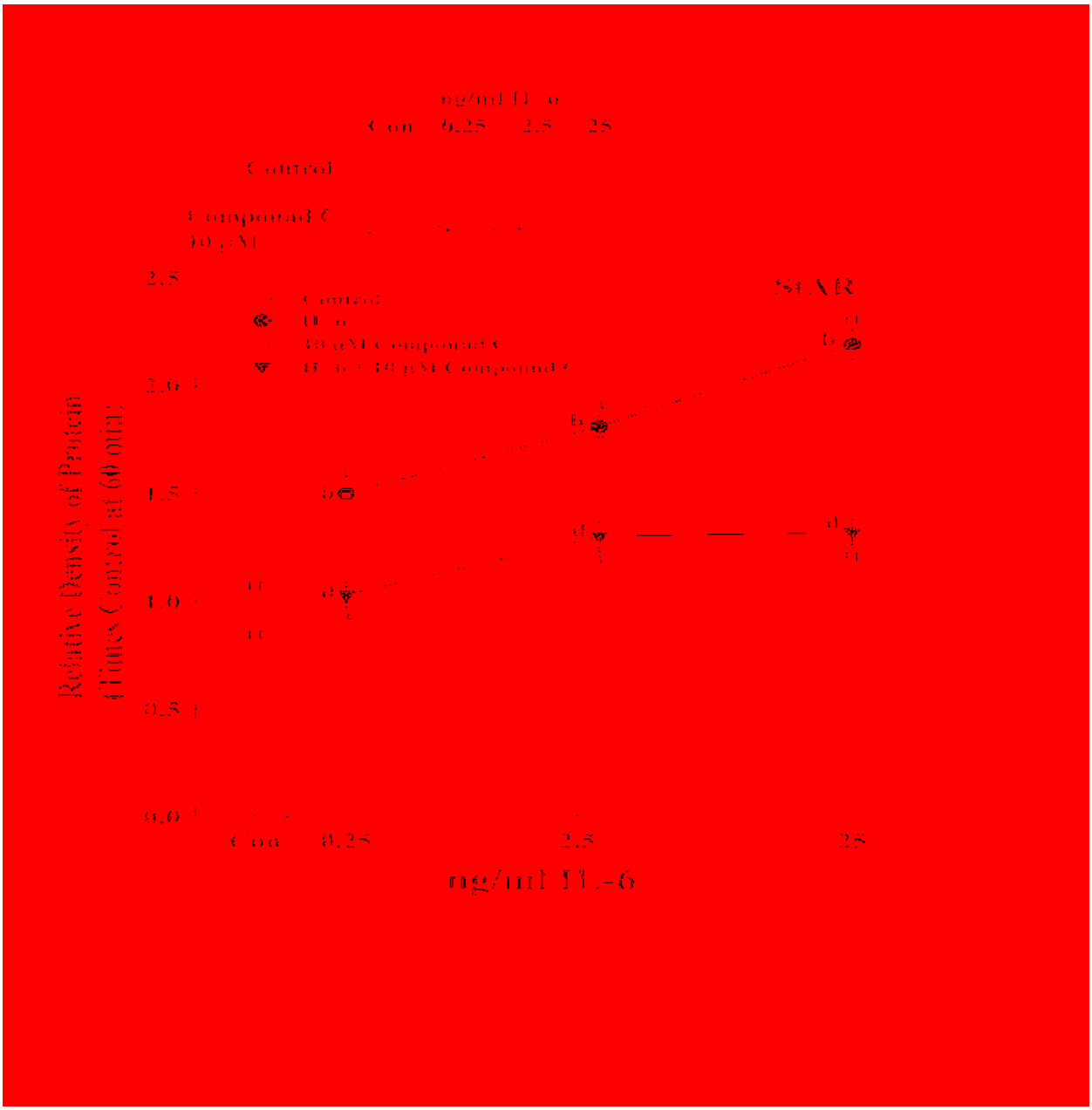

Figure 3-8: The effects of compound C on IL-6-induced StAR protein expression in the ZF

Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium or 10 μM compound C. The incubation medium was then removed and the ZF fragments were exposed for 60 minutes to RPMI medium as control or 0.25, 2.5, or 25ng/ml IL-6 in the presence or absence of 10 μM compound C. The amount of StAR protein was determined by western blot and the values are expressed as a ratio of the absorbance of the StAR band in each treatment group divided by the absorbance of the control sample at 60 minutes. In the upper section of the figure is illustrated a representative western blot for StAR. The lower section of the figure is the combined results from multiple experiments with each point representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (0.25, 2.5 or 25 ng/ml IL-6)).

CHAPTER 4: INTERLEUKIN-6 INHIBITS THE EXPRESSION OF STAR AND P450SCC IN THE ADRENAL ZONA RETICULARIS THROUGH ACTIVATION OF AMP-ACTIVATED PROTEIN KINASE

Introduction

The zona reticularis (ZR) of the adrenal cortex releases androgens including dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and androstenedione. These androgens affect skeletal muscle, the immune system, the inflammatory system, the central nervous system, the cardiovascular system, body composition, and metabolism [1, 3]. Adrenal androgen release is increased by adrenocorticotrophic hormone (ACTH). However, in addition to ACTH other factors regulate adrenal androgen production, because plasma concentrations of adrenal androgens and ACTH are often not well correlated [2, 3, 4, 6]. During acute stress the plasma concentrations of adrenal androgens increase [2, 3]. In contrast, prolonged inflammatory stress decreases the plasma concentrations of these steroids [3, 7]. Aging also decreases plasma adrenal androgens [8].

The adrenal gland releases factors that may function as autocrine or paracrine mediators of adrenal function, including interleukin-6 (IL-6) [8, 10]. The plasma concentrations of IL-6 and the plasma concentrations of adrenal androgens in humans have an inverse correlation during the latter stages following endotoxin injections and chronic inflammatory diseases [7, 8, 10]. Similarly, during aging the plasma concentration of IL-6 increases in mice, rats, monkeys, and humans, while adrenal androgens decrease [8]. In male humans, the plasma IL-6 concentrations negatively correlate with the plasma concentration of DHEAS [8]. In support of this hypothesis, neutralization of plasma IL-6 with antibodies increases the adrenal androgen plasma concentrations in humans with rheumatoid arthritis [22].

AMP-dependent protein kinase (AMPK) mediates the effects of IL-6 in skeletal muscle [70, 71, 72], and the adrenal zona fasciculata (ZF) (Chapter 3). Previous studies performed in our laboratory demonstrated that IL-6 decreases bovine ZR adrenal androgen release [14, 16]. Thus, IL-6 is hypothesized to play a role in the regulation of adrenal steroidogenesis in the ZR. The IL-6 inhibition of androgen release from ZR cells is accompanied by a reduction in the expression of the ZR steroidogenic proteins, steroidogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (P450scc), and a decrease in expression of the nuclear factor steroidogenic factor-1 (SF-1) that enhances steroidogenesis [16]. In contrast, IL-6 increases the expression of dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-1) [16]. DAX-1 is a nuclear factor that inhibits the expression of steroidogenic enzymes in the ZR. Since SF-1 is stimulatory and DAX-1 is inhibitory in steroidogenesis, the increase in DAX-1 and the decrease in SF-1 may be the underlying cause for the IL-6 induced decrease in StAR and P450scc in the ZR. Although AMPK may mediate the effects of IL-6 in skeletal muscle and the adrenal ZF, the role of AMPK in the adrenal ZR is unclear. Thus, it was hypothesized that similar to the adrenal ZF; AMPK may be responsible for the inhibition of StAR and P450scc by IL-6 in the adrenal ZR. Furthermore, it was hypothesized that AMPK activation may be responsible for the increased expression of DAX-1 and decreased expression of SF-1 in the ZR.

Results

AICAR Effects on AMPK Activation in the ZR

The AMPK activator AICAR was initially tested for its ability to activate AMPK in the adrenal ZR tissue fragments. AICAR dose-response and time-course experiments were performed as described in Materials and Methods, and protein levels of PACC and PAMPK in

response to AICAR stimulation were measured using western blots. Phosphorylation of AMPK is an indicator of AMPK activation [68]. The protein expression level of PAMPK increased in both the dose-response (Fig. 4-1-C) and time-course (Fig. 4-1-D) experiments. AMPK activates acetyl-CoA-carboxylase (ACC) by phosphorylation [68]. Therefore PACC protein levels are an index of the activity of AMPK. The expression of PACC increased in both the dose-response (Fig. 4-1-A) and time-course (Fig. 4-1-B) experiments. Accordingly these results demonstrate both a dose-dependent and time-dependent increase in the levels of PACC and PAMPK in response to AICAR stimulation. These observations suggested that AICAR increased AMPK activation in the adrenal ZR.

IL-6 Effects on AMPK Activation in the ZR

The effects of IL-6 on AMPK activation in adrenal ZR tissue fragments were investigated in order to determine if IL-6 may regulate adrenal steroidogenesis in the ZR through AMPK activation. IL-6 dose-response and time-course experiments were performed as described in Materials and Methods, and the protein levels of PACC and PAMPK in response to IL-6 stimulation were measured by western blots. The expression of PAMPK increased in both dose-response (Fig. 4-2-C) and time-course (Fig. 4-2-D) experiments in response to IL-6 treatment. Furthermore, in response to IL-6 treatment the expression of PACC increased in both dose-response (Fig. 4-2-A) and time-course (Fig. 4-2-B) experiments. These results provide evidence that IL-6 increases AMPK activation in the adrenal ZR and therefore support the hypothesis that IL-6 could cause downstream changes in adrenal steroidogenesis through an AMPK-dependent pathway.

Effects of AMPK Activation on Steroidogenic Factors in the ZR

Since the above experiments demonstrated that AICAR stimulates AMPK activation in the adrenal ZR, the effects of AMPK activation on the adrenal steroidogenic factors StAR, P450scc, DAX-1 and SF-1 were determined. AICAR dose-response and time-course experiments were performed in adrenal ZR tissue fragments as described in Materials and Methods. Protein levels of StAR, P450scc, DAX-1 and SF-1 were measured using western blots and mRNA levels of these steroidogenic proteins were measured using standard RT-PCR.

As StAR transports cholesterol into the mitochondrion and controls the rate limiting step in adrenal steroidogenesis, the mRNA and protein expression of StAR are predictors of changes in adrenal steroidogenesis [26, 27, 28]. StAR mRNA expression decreased in both the dose-response (Fig. 4-3-A) and time-course (Fig. 4-3-C) experiments. Likewise, the expression of StAR protein decreased in both the dose-response (Fig. 4-3-B) and time-course (Fig. 4-3-D) experiments in response to AICAR treatment. Therefore increased AMPK activation results in decreased StAR expression in adrenal ZR tissue.

P450scc expression is also an index of steroidogenesis since P450scc is the enzyme which cleaves cholesterol into pregnenolone [30]. The mRNA expression of P450scc decreased in both the dose-response (Fig. 4-4-A) and time-course (Fig. 4-4-C) experiments in response to AICAR treatment. Similarly, protein expression of P450scc decreased in both the dose-response (Fig. 4-4-B) and time-course (Fig. 4-4-D) experiments in response to AICAR treatment. Thus, the results demonstrate that increased AMPK activation decreased P450scc expression in adrenal ZR tissue.

Steroidogenic factor-1 (SF-1) is a transcription factor that up-regulates adrenal steroidogenesis, and therefore the expression of SF-1 is a predictor of adrenal steroidogenesis

[31, 32, 34, 35]. AICAR decreased the mRNA expression of SF-1 in both the dose-response (Fig. 4-5-A) and time-course (Fig. 4-5-C) experiments. Similarly, AICAR decreased the expression of SF-1 protein in the ZR tissue in both the dose-response (Fig. 4-5-B) and time-course (Fig. 4-5-D) experiments. Therefore increased AMPK activation decreased the expression of the stimulatory transcription factor SF-1 in the adrenal ZR.

DAX-1 is an inhibitory transcription factor which down-regulates adrenal steroidogenesis. Higher levels of DAX-1 correlate with inhibition of adrenal ZR steroidogenesis, whereas lower levels of DAX-1 correlate with increased stimulation of steroidogenesis [37, 38, 39, 40, 41, 42, 43]. The mRNA expression of DAX-1 increased in both the dose-response (Fig. 4-6-A) and time-course (Fig. 4-6-C) experiments in response to AICAR treatment. Likewise, AICAR increased the expression of DAX-1 protein in the dose-response (Fig. 4-6-B) and time-course (Fig. 4-6-D) experiments. Therefore, AMPK activation increased the expression of DAX-1 in the adrenal ZR.

Effects of AMPK Inhibition on Steroidogenic Factors in the ZR

Since the previous experiments indicated that AMPK activation by AICAR and IL-6 leads to decreases in StAR, P450scc, and SF-1, and an increase in DAX-1 in the adrenal ZR tissue, experiments were performed with the AMPK inhibitor compound C to determine if these changes were mediated via AMPK activation. Adrenal ZR tissue fragments were treated with compound C alone, IL-6 alone, AICAR alone, IL-6 and compound C, and AICAR and compound C. The experiments were performed as explained in Materials and Methods.

In order to confirm that compound C inhibits AMPK activation in ZR tissue, western blots were conducted for PACC and PAMPK in ZR tissue treated with compound C. Compound C significantly reduced the elevated expression of PACC protein (Fig. 4-7-E) and PAMPK

protein (Fig. 4-7-F) induced by exposure to AICAR in the ZR tissue. Likewise compound C significantly reduced the expression of PACC protein (Fig. 4-7-E) and PAMPK protein (Fig. 4-7-F) induced by IL-6. Thus compound C inhibits AMPK activation in the ZR. It was previously demonstrated that IL-6 decreases the expression of StAR, P450scc, and SF-1 in the ZR tissue [14, 16]. Compound C significantly blocked the inhibition of the expression of StAR protein (Fig. 4-7-A), P450scc protein (Fig. 4-7-B), and SF-1 protein (Fig. 4-7-C) induced by AICAR or IL-6. Compound C significantly reversed the stimulation of DAX-1 protein expression (Fig. 4-7-D) mediated by AICAR or IL-6 exposure. Therefore, AMPK activation may be responsible for the IL-6 induced inhibition of the expression of StAR, P450scc, and SF-1, and stimulation of expression of DAX-1 in the adrenal ZR. However it is possible that IL-6 inhibition of the steroidogenic proteins is only mediated by AMPK at high concentrations of IL-6, therefore the effect of compound C on different concentrations of IL-6 was assessed. Adrenal ZR tissue was exposed to IL-6 at 0.25, 2.5, or 25 ng/ml in the presence or absence of 10 μM compound C, and StAR protein expression was determined by western blotting. StAR protein expression was inhibited in a concentration-dependent manner by IL-6, and the inhibition of StAR expression was reversed by compound C at all concentrations of IL-6 (Fig. 4-8). Thus IL-6 is possibly inhibiting the expression of steroidogenic enzymes through activation of AMPK over a wide range of IL-6 concentrations.

Discussion

The results of this study support a role for AMPK in regulating steroidogenesis in the adrenal ZR. AMPK activation by AICAR modifies steroidogenic protein expression by inhibiting the expression of StAR and P450scc protein and mRNA. StAR and P450scc expression are the rate limiting steps in steroidogenesis, with StAR expression regulating the

acute phases of steroidogenesis [26, 27, 28], and P450scc expression being involved in the more chronic aspects of steroidogenesis regulation [30]. The AICAR inhibition of StAR and P450scc expression may be in part mediated through SF-1 and DAX-1, because AICAR decreases the expression of SF-1 and increases the expression of DAX-1 in the ZR. SF-1 stimulates the expression of proteins promoting steroidogenesis, whereas DAX-1 inhibits the expression of these proteins. In support of the hypothesis that AICAR is modifying the expression of the steroidogenic proteins through AMPK activation, AICAR in a dose-dependent and time-dependent manner increased the expression of PAMPK and PACC, two indicators of AMPK activation. Furthermore, the AMPK inhibitor compound C blocked the AICAR-induced stimulation of AMPK and ACC phosphorylation, and also reversed AICAR mediated decreased expression of StAR, P450scc, and SF-1, and increased expression of DAX-1.

The IL-6 inhibition of StAR and P450scc in the adrenal ZR is probably mediated through AMPK activation. This hypothesis is supported by the observation that IL-6 treatment of ZR tissues results in a dose-dependent and time-dependent increase in the expression of PAMPK and PACC (Fig. 4-2). Furthermore, IL-6 in the ZR decreases the expression of StAR, P450scc, and SF-1 and increases the expression of DAX-1 [16]. These changes in StAR, P450scc, SF-1, and DAX-1 are identical to the changes of these proteins following exposure to AICAR (Figs. 4-3, 4-4, 4-5, 4-6). Evidence that IL-6 is functioning through AMPK activation is provided by the experiment in which ZR cells were exposed to IL-6 in the presence or absence of compound C. Compound C blocks the inhibitory and stimulatory effects of IL-6 on the expression of StAR, P450scc, SF-1, and DAX-1 (Fig. 4-7-A, B, C, D). These effects are probably mediated through AMPK inhibition by compound C in that this inhibitor blocked the expression of PAMPK and PACC (Fig. 4-7-E, F). Compound C blocked the IL-6-mediated inhibition of StAR expression at

IL-6 concentrations ranging from 0.25 ng/ml to 25 ng/ml indicating that AMPK is mediating the decrease in StAR expression over a wide range of IL-6 concentrations (Fig. 4-8). The possibility that compound C is having a nonspecific effect upon the steroidogenic ZR tissue is refuted by the observation that the ACTH-stimulated increase in steroidogenic proteins is not affected by compound C in ZF tissue (Chapter 3). The ZR is only the third tissue after the skeletal muscle, and the adrenal ZF tissue, in which IL-6 has been shown to modify cellular function through an AMPK-dependent mechanism. Although the majority of experiments with IL-6 presented in this study utilized recombinant murine IL-6, selected experiments were repeated with recombinant bovine IL-6 and the data from the bovine IL-6 were identical to the murine IL-6 data (data not presented).

Thus, these results suggest that AMPK activation is necessary for the IL-6-induced inhibition of expression of StAR and P450scc in the ZR. However, it is not exactly known if AMPK activation is sufficient for the inhibition of adrenal androgen release from the ZR. It is possible to hypothesize that the reversal of IL-6-induced StAR and P450scc expression mediated by compound C will result in increased adrenal androgen release. However, proving this hypothesis will require additional experiments in which androgen release is determined from cultured ZR cells in the presence and absence of IL-6 and compound C. The inhibition of StAR and P450scc expression due to IL-6 and AMPK activation by AICAR may be mediated by these agents decreasing the expression of the nuclear factor SF-1 and increasing the expression of the nuclear factor DAX-1. However, it is also possible that other nuclear factors may also be involved in this regulation, as well as any other unidentified intermediates.

In summary, AMPK activation is necessary for IL-6 inhibition of StAR and P450scc in adrenal ZR tissue. Thus AMPK activation by IL-6 and other inflammatory factors may be

responsible for the decreased adrenal androgen release manifested during chronic inflammatory stress. These changes in adrenal steroids have profound metabolic effects. Therefore, the hypothesis that AMPK modifies the adrenal release of steroid hormones is consistent with AMPK functioning as a key regulator of overall metabolism. These findings could have potential implications in treating the complications associated with androgen deficiency and chronic inflammatory stress.

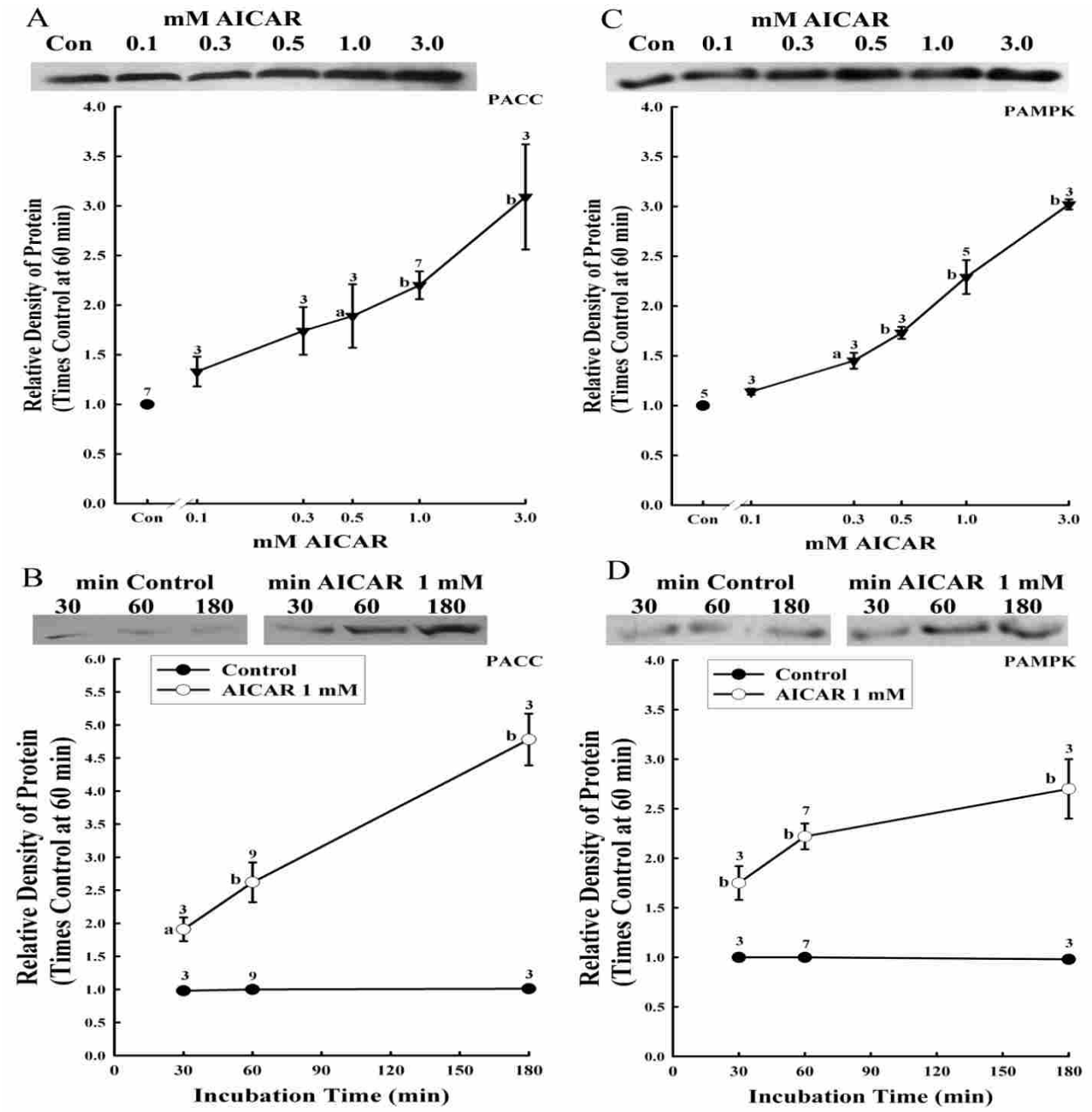

Figure 4-1: The dose-dependent and time-dependent effects of AICAR on the expression of PACC and PAMPK in the ZR

PACC (A, B) or PAMPK (C, D) protein expression was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment protein bands divided by the absorbance of the control treatment bands at 60 min. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiments (A, C):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.1, 0.3, 0.5, 1.0, or 3.0 mM AICAR. **Time course experiments (B, D):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 min. with RPMI medium (Control) or 1 mM AICAR.

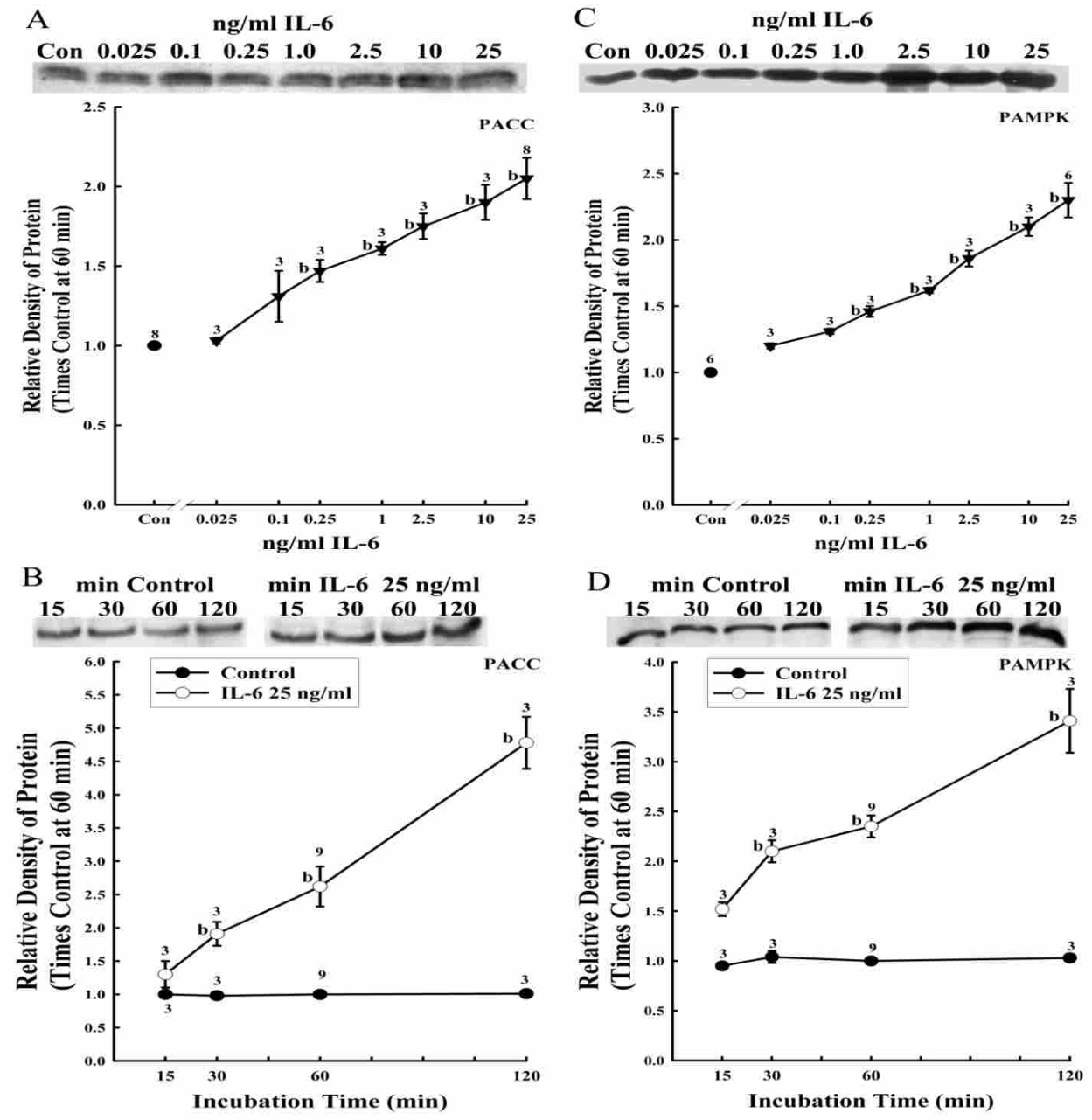

Figure 4-2: The dose-dependent and time-dependent effects of IL-6 on the expression of PACC and PAMPK in the ZR

PACC (A, B) or PAMPK (C, D) protein expression was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment protein bands divided by the absorbance of the control treatment bands at 60 min. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, C):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10, or 25 ng/mL IL-6. **Time-course experiments (B, D):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/mL IL-6.

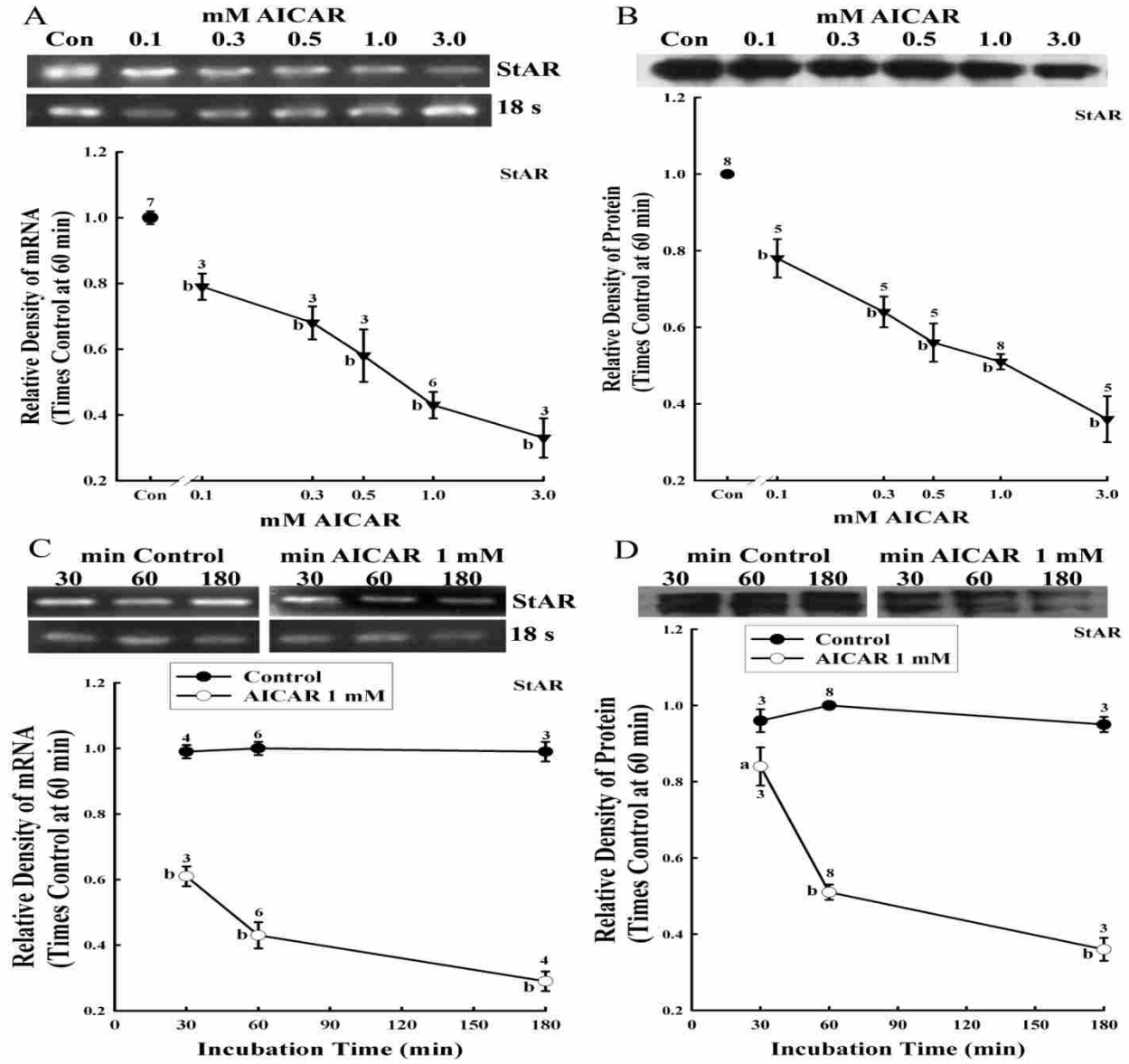

Figure 4-3: The dose-dependent and time-dependent effects of AICAR on the expression of StAR mRNA and protein in the ZR

StAR mRNA expression (A, C) was determined by RT-PCR and StAR protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each StAR band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the StAR mRNA/18s ratio of the control sample at 60 min on the same gel. The StAR protein expression was quantified by determining the ratio of the absorbance of the treatment StAR bands divided by the absorbance of the control treatment StAR bands at 60 min. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

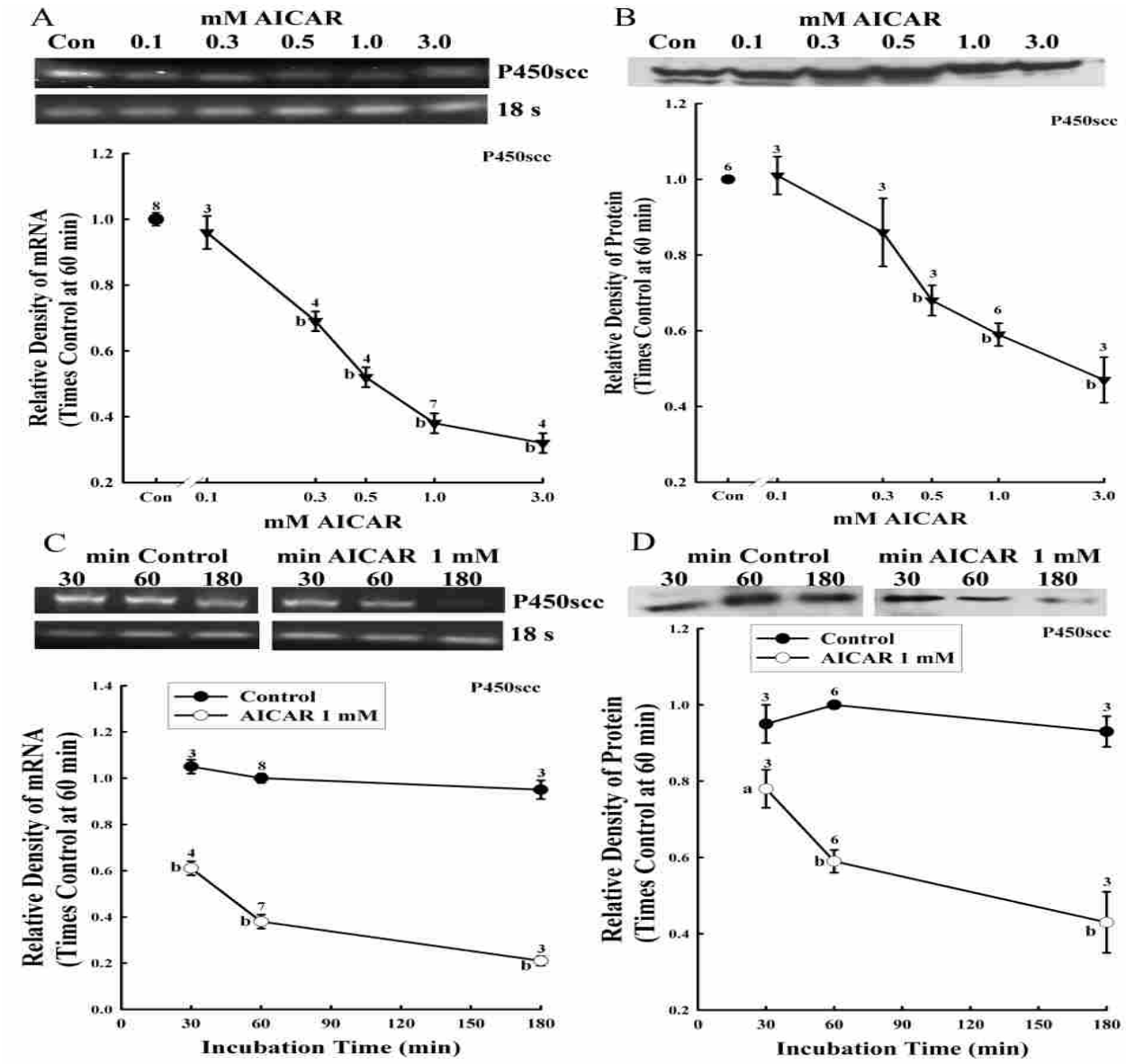

Figure 4-4: The dose-dependent and time-dependent effects of AICAR on the expression of P450scc mRNA and protein in the ZR

P450scc mRNA expression (A, C) was determined by RT-PCR and P450scc protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each P450scc band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the P450scc mRNA/18s ratio of the control sample at 60 minutes on the same gel. The P450scc protein expression was quantified by determining the ratio of the absorbance of the treatment P450scc bands, divided by the absorbance of the control treatment P450scc bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

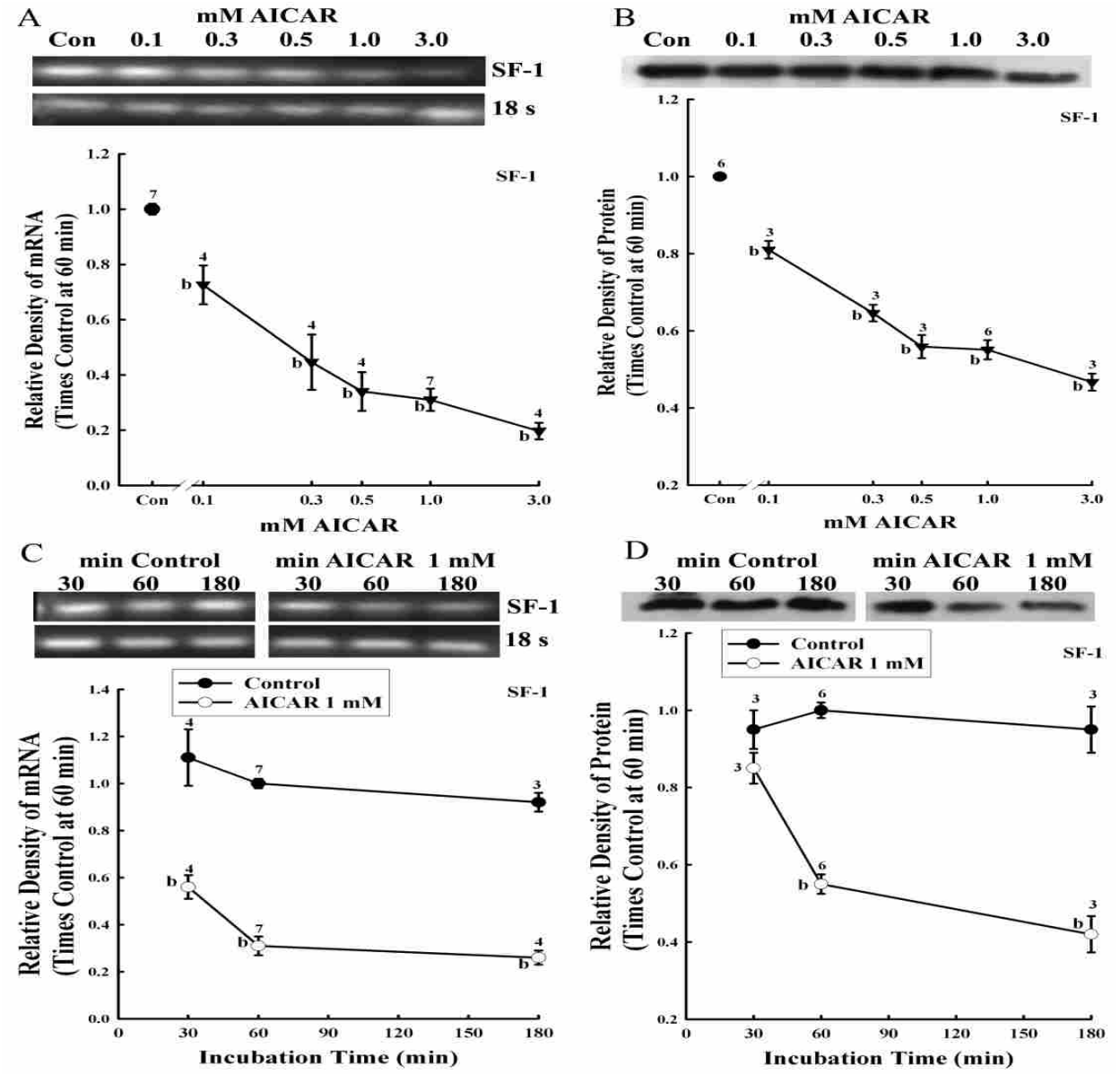

Figure 4-5: The dose-dependent and time-dependent effects of AICAR on the expression of SF-1 mRNA and protein in the ZR

SF-1 mRNA expression (A, C) was determined by RT-PCR, and SF-1 protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each SF-1 band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the SF-1 mRNA/18s ratio of the control sample at 60 minutes on the same gel. The SF-1 protein expression was quantified by determining the ratio of the absorbance of the treatment SF-1 bands divided by the absorbance of the control treatment SF-1 bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

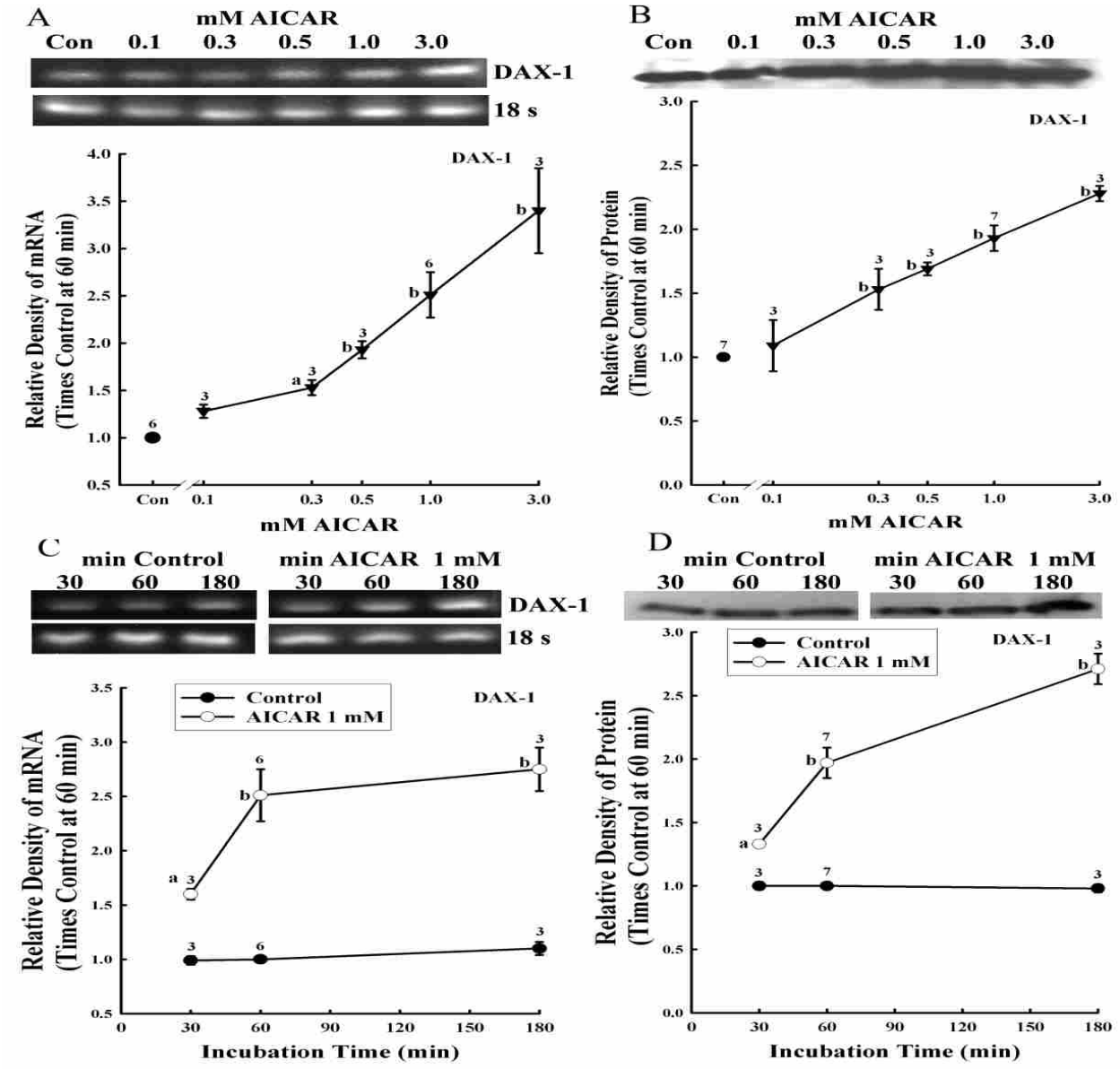

Figure 4-6: The dose-dependent and time-dependent effects of AICAR on the expression of DAX-1 mRNA and protein in the ZR

DAX-1 mRNA expression (A, C) was determined by RT-PCR, and DAX-1 protein expression (B, D) was determined by western blotting. For the mRNA quantification the absorbance of each DAX-1 band on a gel was divided by the absorbance of the corresponding 18S band on the same gel. This value was then normalized by dividing it by the DAX-1 mRNA/18s ratio of the control sample at 60 minutes on the same gel. The DAX-1 protein expression was quantified by determining the ratio of the absorbance of the treatment DAX-1 bands divided by the absorbance of the control treatment DAX-1 bands at 60 minutes. In the upper section of each panel is illustrated representative western or RT-PCR blots. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose response experiments (A, B):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, 0.5 1.0, or 3.0 mM AICAR. **Time-course experiments (C, D):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

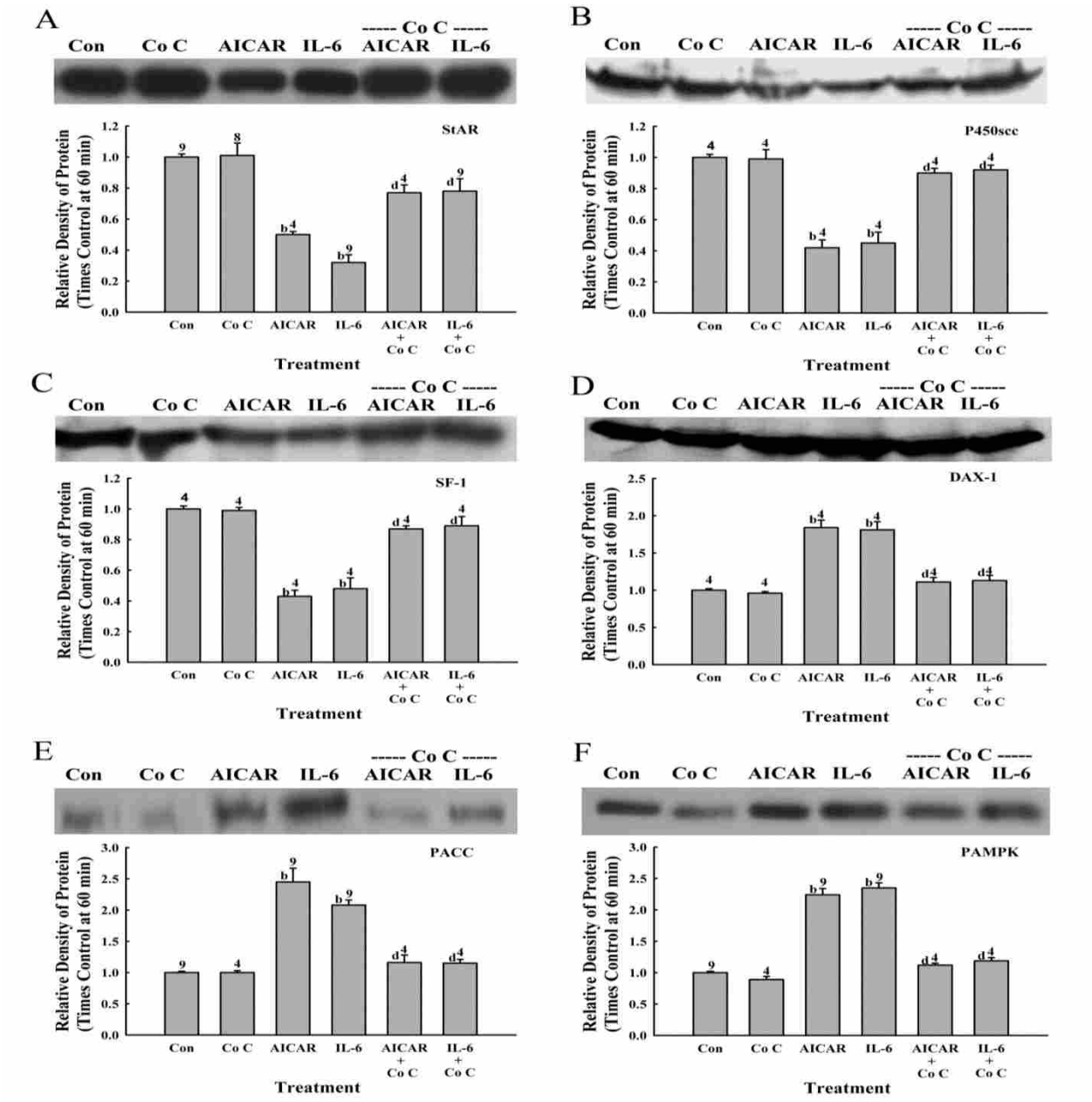

Figure 4-7: The effects of compound C on IL-6-stimulated and AICAR-stimulated expression of steroidogenic factors, PACC and PAMPK in the ZR

Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium or 10 µM compound C. The incubation medium was then removed and the ZR fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR + 10 µM compound C; 25 ng/ml IL-6 + 10 µM compound C. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated representative western blots for (A) StAR; (B) P450scc; (C) SF-1; (D) DAX-1); (E) PACC; (F) PAMPK. The lower section of each panel is the combined results from multiple experiments with the bar representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6)).

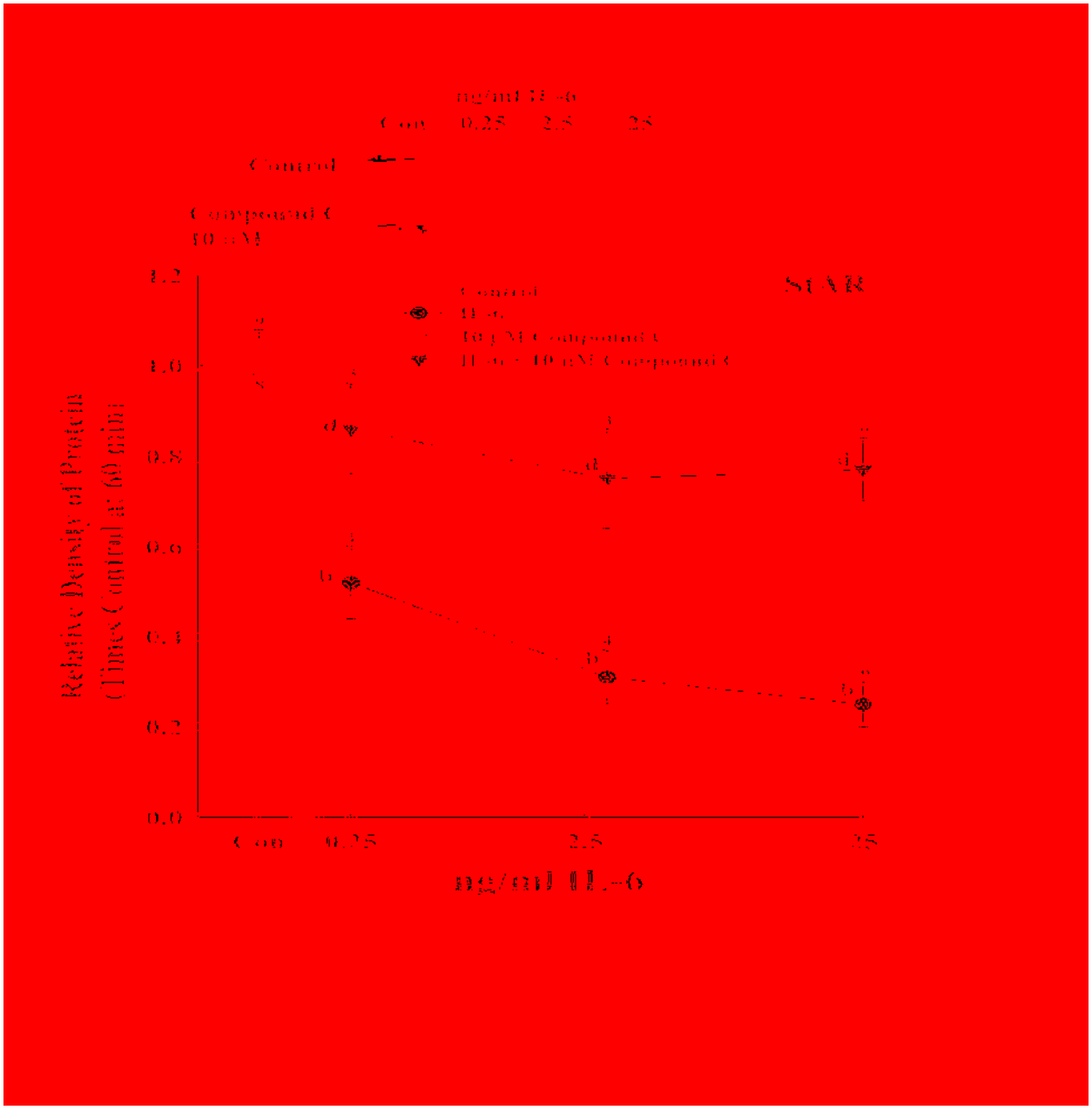

Figure 4-8: The effects of compound C on IL-6-induced StAR protein expression in the ZR

Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium or 10 µM compound C. The incubation medium was then removed and the ZR fragments were exposed for 60 minutes to RPMI medium as control or 0.25, 2.5, or 25 ng/ml IL-6 in the presence or absence of 10 µM compound C. The amount of StAR protein was determined by western blot and the values are expressed as a ratio of the absorbance of the StAR band in each treatment group divided by the absorbance of the control sample at 60 minutes. In the upper section of the figure is illustrated a representative western blot for StAR. The lower section of the figure is the combined results from multiple experiments with each point representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (0.25, 2.5 or 25 ng/ml IL-6).

CHAPTER 5: THE ACTIVATOR PROTEIN-1 COMPLEX FUNCTIONS AS A BIOCHEMICAL INTERMEDIATE IN THE INTERLEUKIN-6 AND AMP-ACTIVATED PROTEIN KINASE REGULATION OF STEROIDOGENIC ENZYMES IN THE ZONA FASCICULATA

Introduction

Interleukin-6 (IL-6) may function as an important mediator of steroidogenesis in the adrenal zona fasciculata (ZF) [1, 8, 10, Chapter 3]. In many tissues, IL-6 functions in part by the activation of the activator protein 1 (AP-1) family of transcription factors [56, 58]. However, the effects of IL-6 on the expression of AP-1 in steroidogenic tissues have not yet been determined. AP-1 is a family of nuclear transcription factors which consists of homo or hetero dimers of JUN family proteins, as well as heterodimers of JUN and FOS family proteins. The three JUN family members (cJUN, JUN B, and JUN D) and the four FOS family members (cFOS, FOS B, FRA-1, and FRA-2) can combine to form 18 different dimeric combinations of AP-1[54, 55]. However the major subunits that are well documented to dimerize, and cause changes in transcription are cFOS, cJUN, JUN B, and JUN D. Moreover the phosphorylation of cJUN enhances the activity of the AP-1 complex [54, 55].

Various secretagogues increase the expression of the AP-1 units in the ovary, testes, and adrenal glands [56, 57, 58]. Preliminary studies in our laboratory have likewise demonstrated that IL-6 increases the expression of AP-1 proteins in the adrenal cortex [16]. AP-1 binds to specific nucleotide sequences on the promoters of various steroidogenic genes and modifies the transcription of the mRNA for steroidogenic proteins [56]. The mechanism through which IL-6 increases the expression of AP-1 proteins may involve IL-6 activation of the Janus kinase/signal

transducers and activator of transcription (JAK/STAT) biochemical pathway. The IL-6 stimulation of the JAK/STAT pathway results in phosphorylated STATs migrating to the nucleus, binding to the promoters of the AP-1 subunit genes, and thereby increasing the transcription of the various proteins that form the AP-1 complex [45, 46]. The increased expression of the AP-1 transcription factors may then result in the increased activation or inhibition of the promoters of the genes involved in steroidogenesis. However the phosphorylated STATs activated by IL-6 may also directly bind to the promoters of key steroidogenic proteins such as steroidogenic acute regulatory protein (StAR) and cholesterol side-chain-cleavage enzyme (P450scc), and to the promoters of nuclear factors such as steroidogenic factor-1 (SF-1) and dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-1).

Additional biochemical pathways may also be involved in the activation of the AP-1 complex. One study performed in AtT20 corticotroph cells provides evidence that AMPK activation can also activate the AP-1 complex [59]. Since IL-6 activates AMPK, it was hypothesized that AMPK increases the expression and phosphorylation of AP-1 proteins, and the AP-1 proteins in turn regulate the expression of steroidogenic proteins in the adrenal ZF. In this study, this hypothesis was tested by determining the effects of IL-6, the AMPK activator AICAR, and the AMPK inhibitor compound C, on the expression of the AP-1 subunits and cJUN phosphorylation. An inhibitor of AP-1, SR11302, was also utilized to determine the role of AP-1 in the expression of the steroidogenic proteins StAR, P450scc, SF-1, and DAX-1.

Results

IL-6 Regulation of AP-1 in the ZF

The role of AP-1 in IL-6 regulation of steroidogenic enzymes in the ZF was investigated by determining if IL-6 affects the expression of the various AP-1 subunits. IL-6 dose-response and time-course experiments were performed on adrenal ZF tissue fragments as described in Materials and Methods. The expression of the AP-1 subunits, cFOS, cJUN, JUN B, and JUN D in response to IL-6 stimulation was measured with western blots. The expression of cFOS (Fig. 5-1-A), cJUN (Fig. 5-2-A), JUN B (Fig. 5-3-A), and JUN D (Fig. 5-4-A) increased in a dose-dependent manner when exposed to various concentrations of IL-6. The expression of these AP-1 subunits was also dependent on the IL-6 incubation time since the expression of cFOS (Fig. 5-1-B), cJUN (Fig. 5-2-B), JUN B (Fig. 5-3-B), and JUN D (Fig. 5-4-B) increased in a time-dependent manner. The phosphorylation of cJUN stimulates the activation of the AP-1 transcription complex [52]. IL-6 dose-dependently (Fig. 5-5-A) and time-dependently (Fig. 5-5-B) increased the expression of PcJUN in the ZF tissue fragments. Thus IL-6 increased the phosphorylation of cJUN and increased AP-1 subunit expression.

Effects of AMPK Activation on AP-1 in the ZF

Since IL-6 increases AP-1 subunit expression and cJUN phosphorylation in a dose-dependent and a time-dependent manner, and IL-6 activates AMPK in ZF tissue (Chapter 3), the effects of AMPK activation on AP-1 subunit expression in the adrenal ZF tissue fragments were determined. Dose-response and time-course experiments were performed on adrenal ZF tissue fragments exposed to the AMPK activator, 5-amino imidazole-4-carboxamide riboside (AICAR), to assess the effects of AMPK activation on AP-1 subunit expression. The expression of the AP-1 subunits, cFOS, cJUN, JUN B, and JUN D in response to AICAR stimulation was

measured using western blots. The expression of cFOS (Fig. 5-6-A), cJUN (Fig. 5-7-A), JUN B (Fig. 5-8-A), and JUN D (Fig. 5-9-A) increased in the dose-response experiments in reaction to increasing concentrations of AICAR. The expression of cFOS (Fig. 5-6-B), cJUN (Fig. 5-7-B), JUN B (Fig. 5-8-B), and JUN D (Fig. 5-9-B) also increased in time-course experiments in response to increasing incubation times in AICAR. These results are consistent with the idea that AMPK activation stimulates the expression of AP-1 subunits in the ZF. Moreover, since cJUN phosphorylation enhances the activation of the AP-1 transcription complex, the expression of phospho-cJun (PcJUN) in response to AICAR exposure was determined. AICAR dose-dependently (Fig. 5-10-A) and time-dependently (Fig. 5-10-B) increased PcJUN expression in the ZF tissue fragments. Thus, AMPK activation increased the activation of AP-1 subunit PcJUN, in addition to increasing the AP-1 protein subunit expression. These results support the hypothesis that AP-1 expression is downstream of AMPK activation in the biochemical pathways involved in IL-6 regulation of steroidogenesis.

Effects of AMPK Inhibition on AP-1 in the ZF

As the previous experiments provides evidence that AMPK activation leads to increases in the expression of AP-1 subunits and phosphorylation of cJUN, experiments were performed with compound C, an AMPK inhibitor, to confirm that AMPK activation was involved in the AP-1 pathway in ZF tissue. Compound C alone had no significant effect on the expression of cFOS (Fig. 5-11-C), cJUN (Fig. 5-11-D), JUN B (Fig. 5-11-A), JUN D (Fig. 5-11-B) and PcJUN (Fig. 5-11-E) as compared to the RPMI medium control treatment. Incubation in IL-6 and AICAR significantly increased the expression of cFOS (Fig. 5-11-C), cJUN (Fig. 5-11-D), JUN B (Fig. 5-11-A), JUN D (Fig. 5-11-B) and PcJUN (Fig. 5-11-E) as compared to the control treatment. Co-incubation with compound C and AICAR or IL-6 significantly decreased the

expression of cFOS (Fig. 5-11-C), cJUN (Fig. 5-11-D), JUN B (Fig. 5-11-A), JUN D (Fig. 5-11-B) and PcJUN (Fig. 5-11-E) induced by AICAR or IL-6 in the ZF tissue. Thus these results provide evidence that AMPK activation by AICAR or IL-6 mediated the increase of AP-1 subunit expression and phosphorylation of cJUN in ZF tissue fragments.

Effects of AP-1 Inhibition on Steroidogenic Factors and AMPK Activation in the ZF

AP-1 was functionally inhibited in a series of experiments using SR11302 in order to determine the role of AP-1 in regulating the adrenal steroidogenic factors StAR, P450scc, SF-1 and DAX-1. SR11302 is an AP-1 inhibition specific retinoid, which is a functional AP-1 inhibitor [63, 64]. The adrenal ZF tissue fragments were exposed to SR11302 as described in Materials and Methods and western blots were performed to quantify the expression of StAR, P450scc, SF-1, and DAX-1 in the ZF tissue. SR11302 alone had no significant effect on the expression of StAR (Fig. 5-12-A), P450scc (Fig. 5-12-B), SF-1 (Fig. 5-12-C) and DAX-1 (Fig. 5-12-D) as compared to control treatments. IL-6 and AICAR treatments significantly increased the expression of StAR (Fig. 5-12-A), P450scc (Fig. 5-12-B), SF-1 (Fig. 5-12-C), and significantly decreased the expression of DAX-1 (Fig. 5-12-D) as compared to the control treatments. Co-incubation with SR11302 and AICAR or IL-6 significantly decreased the expression of StAR (Fig. 5-12-A), P450scc (Fig. 5-12-B), SF-1 (Fig. 5-12-C) and significantly increased the expression of DAX-1 (Fig. 5-12-D) as compared to the AICAR or IL-6 treatments.

Furthermore in additional experiments ZF tissue was co-incubated in SR11302 and IL-6 or AICAR to clarify the relationship between AP-1 and AMPK activation. It can be assumed that if AP-1 is downstream from AMPK activation in the biochemical pathway involved in AICAR and IL-6 modification of steroidogenic proteins, then SR11302 should not modify the IL-6 or AICAR activation of AMPK. The phosphorylation of AMPK (PAMPK) is an index of AMPK

activation, and activated AMPK phosphorylates the enzyme actyl CoA carboxylase (ACC) to form phosphorylated-ACC (PACC), and PACC is also an index of AMPK activation [68]. SR11302 treatment had no significant effect on the AICAR or IL-6 induced increase of PACC (Fig. 5-12-E) and PAMPK (Fig. 5-12-F). Thus AP-1 did not affect AMPK activation in the ZF.

Discussion

IL-6 increases the expression of AP-1 subunits and activates AP-1 in several tissues [57, 58]. However, to our knowledge this is the first study that has demonstrated that IL-6 induces the expression of the AP-1 subunits cFOS, cJUN, JUN B, and JUN D in steroidogenic tissues. Furthermore, IL-6 increases the phosphorylation of cJUN in the ZF tissue, and this phosphorylation increases the activity of the AP-1 complex. The cytokine IL-6 increases the activity of the enzyme AMPK in skeletal muscle, the adrenal ZF, and the adrenal ZR. In these tissues, AMPK activation plays an important role in mediating the biochemical effects of IL-6 further downstream. In one previous study performed in AtT20 corticotroph cells, AMPK activation was demonstrated to increase the activity of the AP-1 complex [59]. Therefore the above study investigated if AMPK activation enhanced the expression of AP-1 subunits and cJUN phosphorylation in the ZF tissue.

The AMPK activator AICAR increased the expression of cFOS, cJUN, JUN B, and JUN D, and increased the phosphorylation of cJUN in a concentration-dependent and time-dependent manner. To our knowledge, this is the second study demonstrating that AMPK activation modulates the expression of AP-1 subunits, and also this is the first study demonstrating that AMPK activation increases the phosphorylation of cJUN. These effects of AICAR on AP-1 subunit expression and cJUN phosphorylation are not mediated by non-specific effects of AICAR, because these effects were completely blocked by exposing the ZF tissue to the AMPK

inhibitor compound C. In support of the hypothesis that IL-6 increases AMPK activity which in turn regulates the expression of the AP-1 subunits and cJUN phosphorylation, compound C blocks the IL-6-induced expression of the AP-1 subunits and cJUN phosphorylation. Therefore, IL-6 increases AMPK activation, which in turn increases the expression of the AP-1 subunits and cJUN phosphorylation.

AP-1 binds to the promoters for StAR and P450scc, and can activate the transcription for these steroidogenic proteins. Inhibition of AP-1 activity by SR11302 blocks the IL-6-induced and AICAR-induced increase in the expression of StAR (Fig. 5-12-A) and P450scc (Fig. 5-12-B). The SR11302 effects on the steroidogenic enzymes cannot be explained by a non-specific effect of SR11302 on AMPK activation because SR11302 has no significant effect on the phosphorylation of AMPK (Fig. 5-12-F) or ACC (Fig. 5-12-E) increased by AICAR or IL-6. Furthermore, the increased DAX-1 protein expression in response to co-incubation with SR11302 and AICAR or IL-6 (Fig. 5-12-D), suggested that the ZF tissue is not poisoned by SR11302. These results together with the relationship between AMPK and AP-1 permit the following biochemical pathway to be hypothesized. IL-6 increases the activity of AMPK, which in turn leads to the expression of AP-1 subunits and the phosphorylation of cJUN. The increased expression of AP-1 subunits and cJUN phosphorylation causes formation of activated AP-1 complexes that in turn bind to the promoters for the StAR and P450scc genes. The binding of the AP-1 complexes results in an activation of the transcription of the StAR and P450scc genes that in turn causes increased expression of StAR and P450scc protein.

Moreover it is important to emphasize that the above described biochemical mechanism of activation of the StAR and P450scc genes does not exclude the possibility that other nuclear factors such as SF-1 and DAX-1 are also involved in the IL-6 activation of steroidogenic gene

expression. Also it is noteworthy that SF-1 expression is increased by IL-6 and SF-1 forms complexes with AP-1 to enhance expression of the StAR and P450scc genes [73]. In contrast, DAX-1 is decreased by IL-6 and DAX-1 generally inhibits the expression of the mRNA of steroidogenic proteins. The specific mechanism by which IL-6 stimulates SF-1 expression and inhibits DAX-1 expression in the ZF is currently unknown. There is no evidence that the SF-1 or DAX-1 promoter has AP-1 binding sequences. Therefore, although the effects of IL-6 on SF-1 and DAX-1 expression are blocked by the AP-1 inhibitor SR11302, it is unlikely that the AP-1 complex is directly increasing the expression of SF-1 and inhibiting the expression of DAX-1. Thus the AP-1 complex is probably indirectly modifying the expression of SF-1 and DAX-1 by changing the expression of other factors that in turn regulate the expression of SF-1 and DAX-1 proteins.

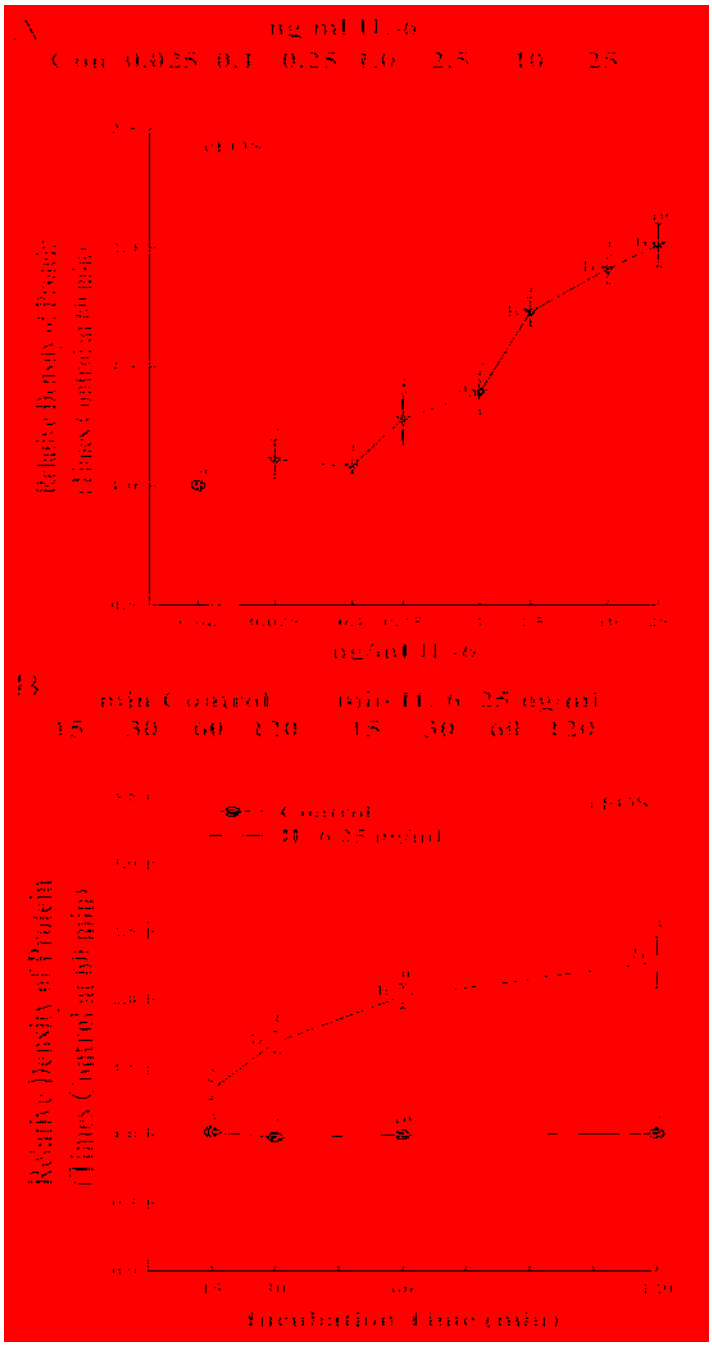

Figure 5-1: The dose-dependent and time-dependent effects of IL-6 on the expression of cFOS in the ZF.

The expression of cFOS was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cFOS bands divided by the absorbance of the control cFOS bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

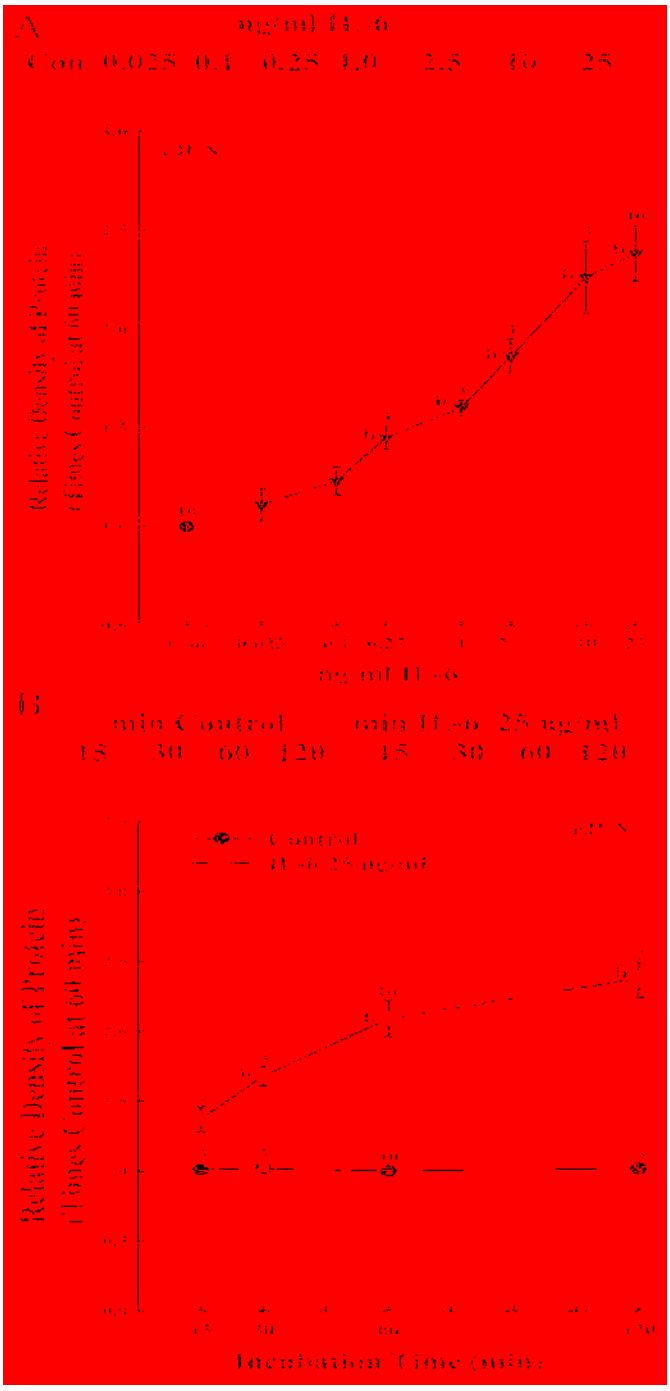

Figure 5-2: The dose-dependent and time-dependent effects of IL-6 on the expression of cJUN in the ZF.

The expression of cJUN was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cJUN bands divided by the absorbance of the control cJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

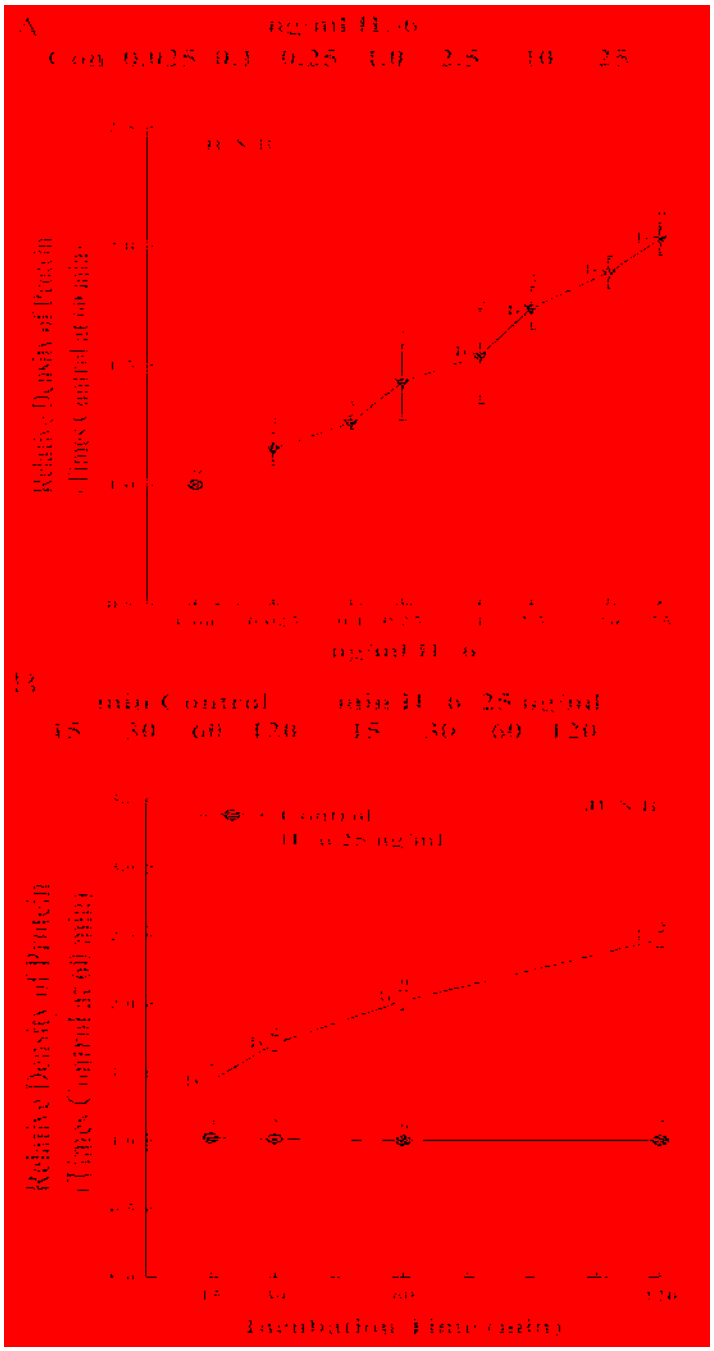

Figure 5-3: The dose-dependent and time-dependent effects of IL-6 on the expression of JUN B in the ZF.

The expression of JUN B was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN B bands divided by the absorbance of the control JUN B bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

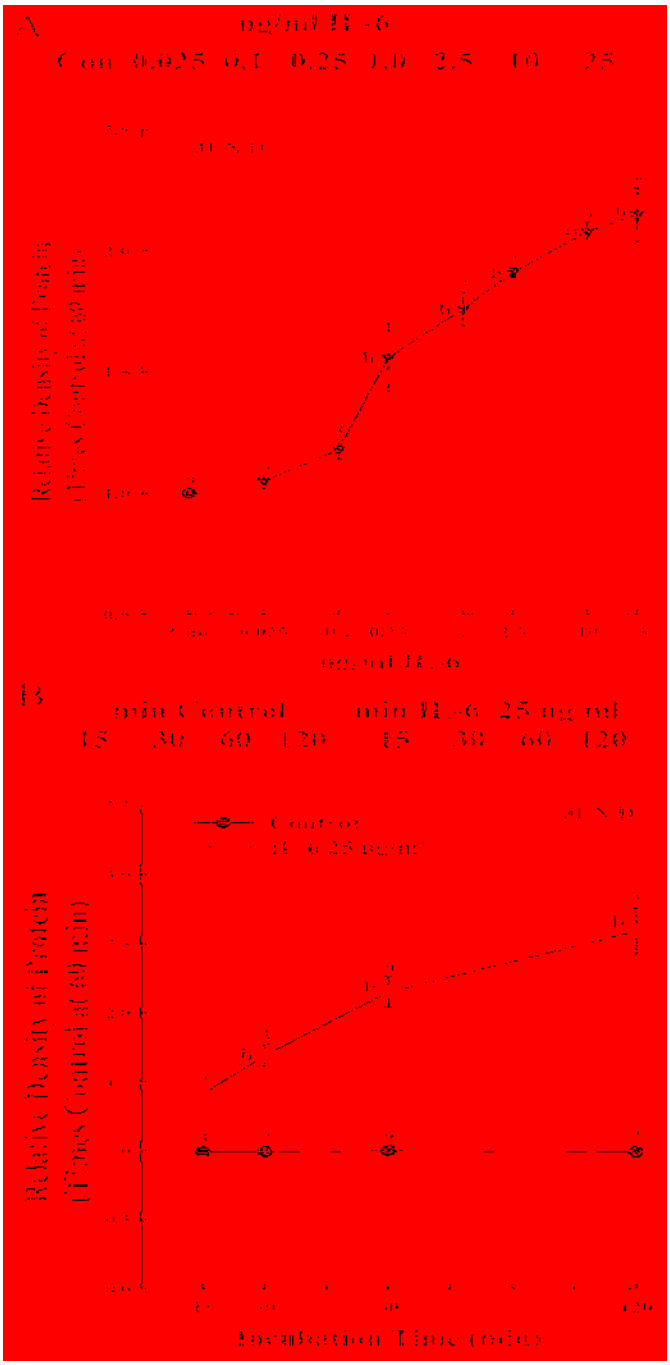

Figure 5-4: The dose-dependent and time-dependent effects of IL-6 on the expression of JUN D in the ZF.

The expression of JUN D was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN D bands divided by the absorbance of the control JUN D bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

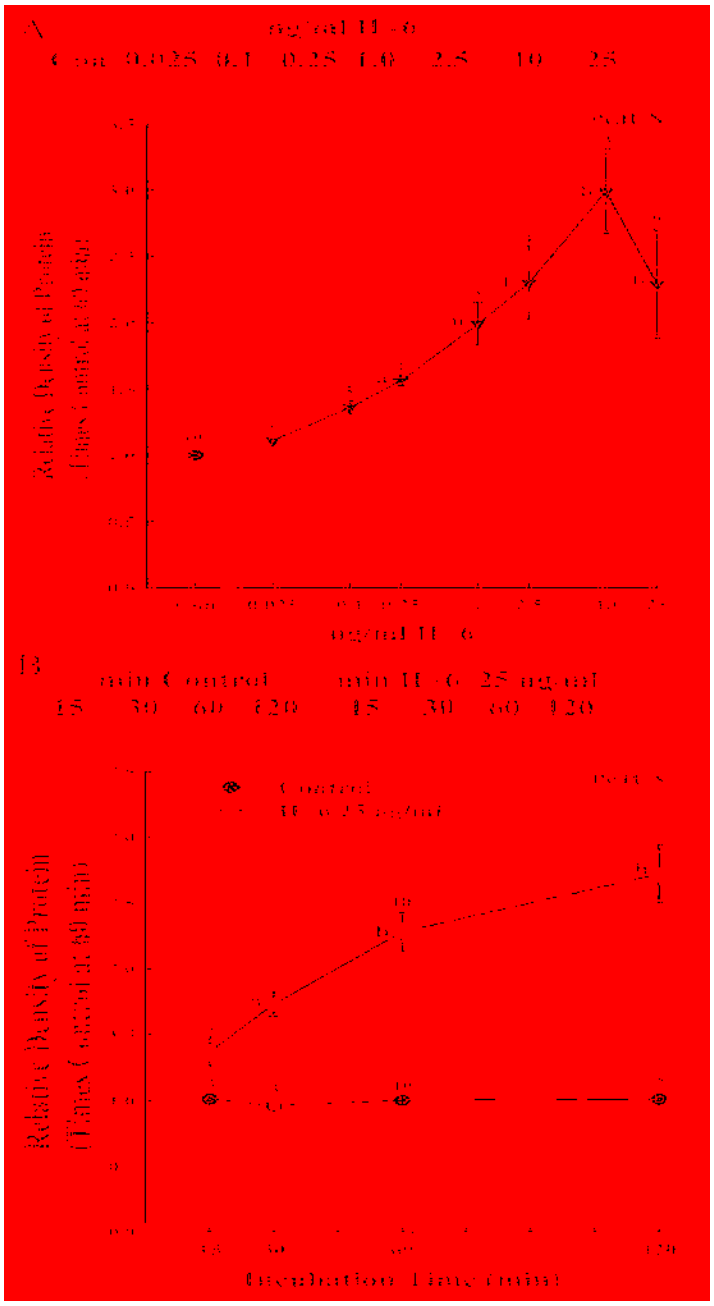

Figure 5-5: The dose-dependent and time-dependent effects of IL-6 on the phosphorylation of cJUN in the ZF.

Phosphorylated cJUN (PcJUN) content was determined utilizing western blots and the values expressed as a ratio of the absorbance of the treatment PcJUN bands divided by the absorbance of the control PcJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZF tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

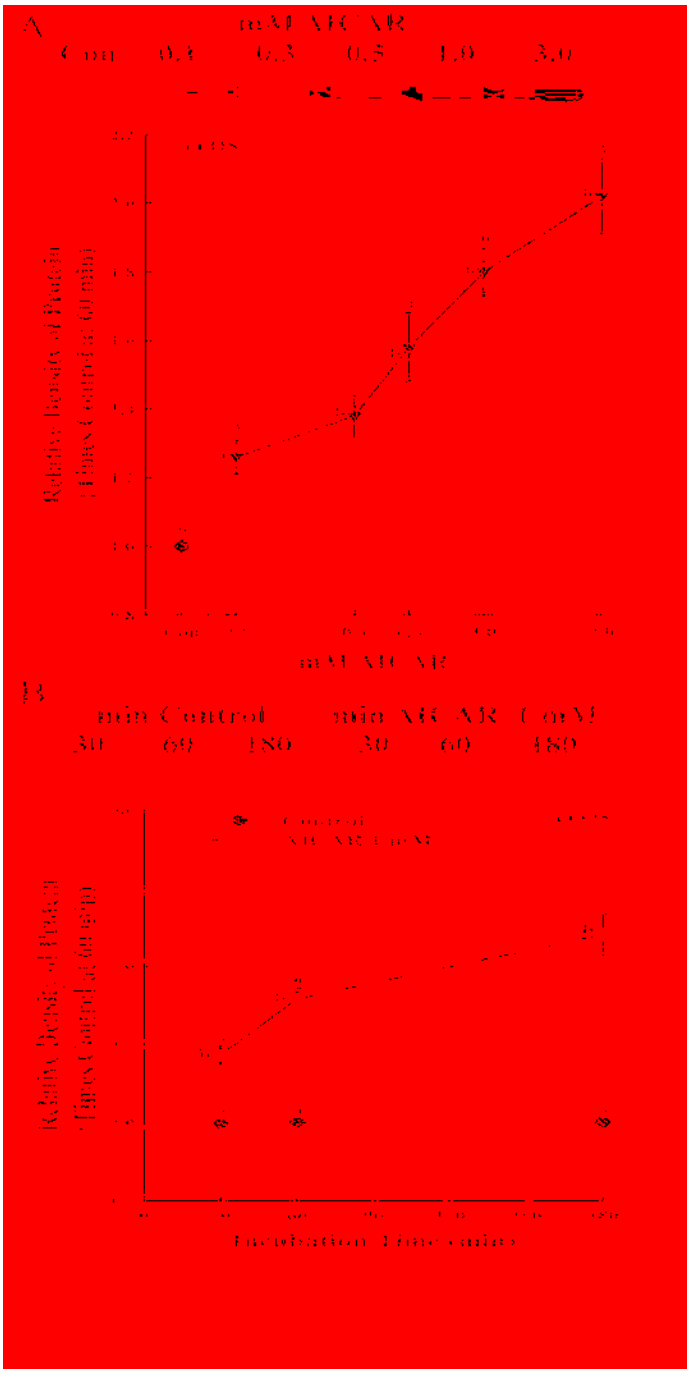

Figure 5-6: The dose-dependent and time-dependent effects of AICAR on the expression of cFOS in the ZF.

The expression of cFOS was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cFOS bands divided by the absorbance of the control cFOS bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

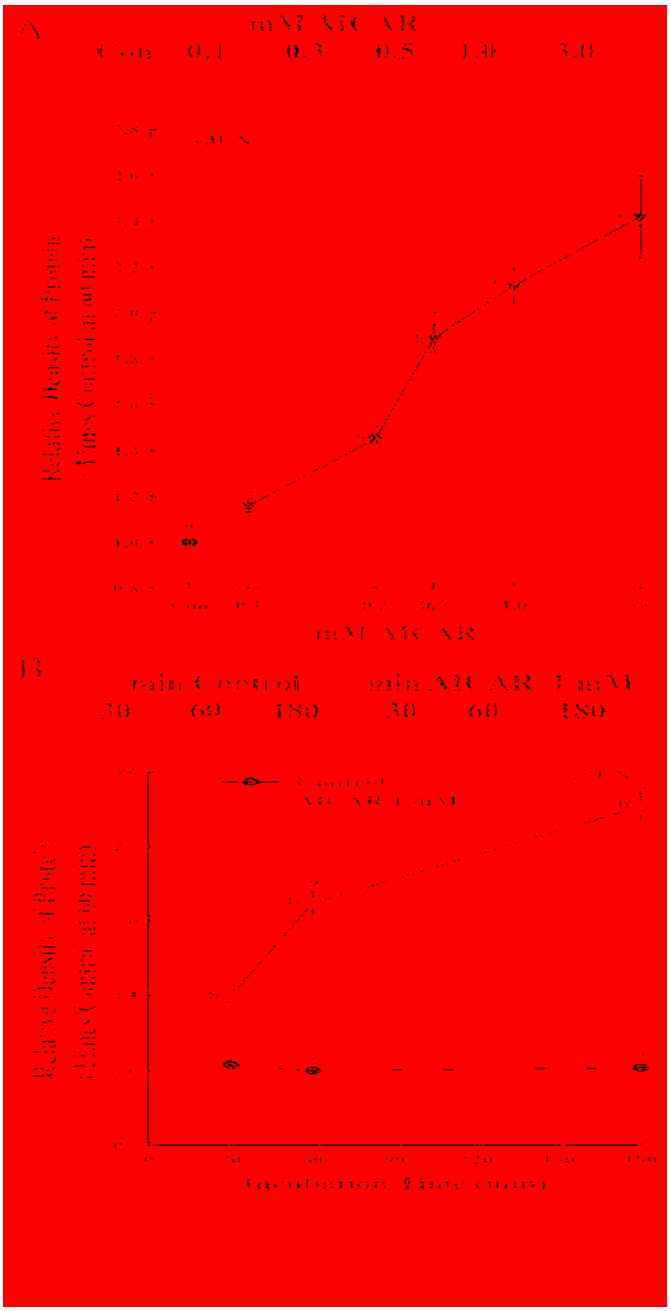

Figure 5-7: The dose-dependent and time-dependent effects of AICAR on the expression of cJUN in the ZF.

The expression of cJUN was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cJUN bands divided by the absorbance of the control cJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

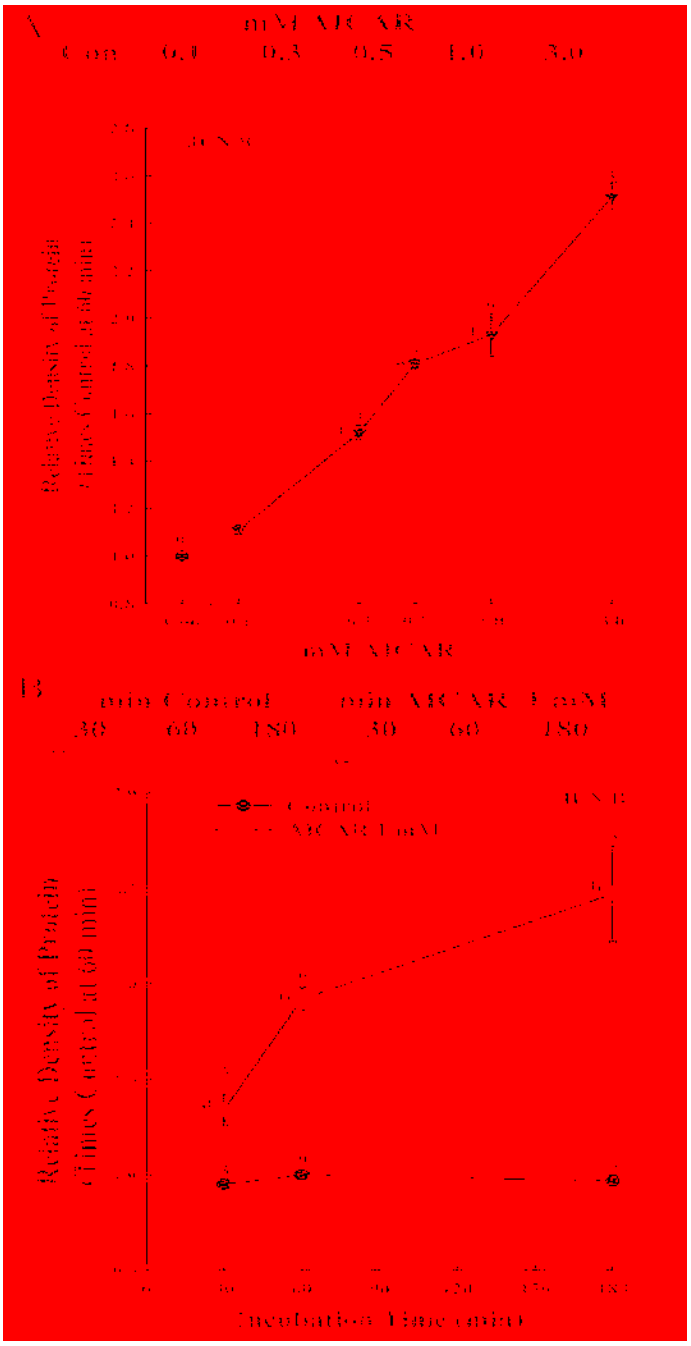

Figure 5-8: The dose-dependent and time-dependent effects of AICAR on the expression of JUN B in the ZF.

The expression of JUN B was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN B bands divided by the absorbance of the control JUN B bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

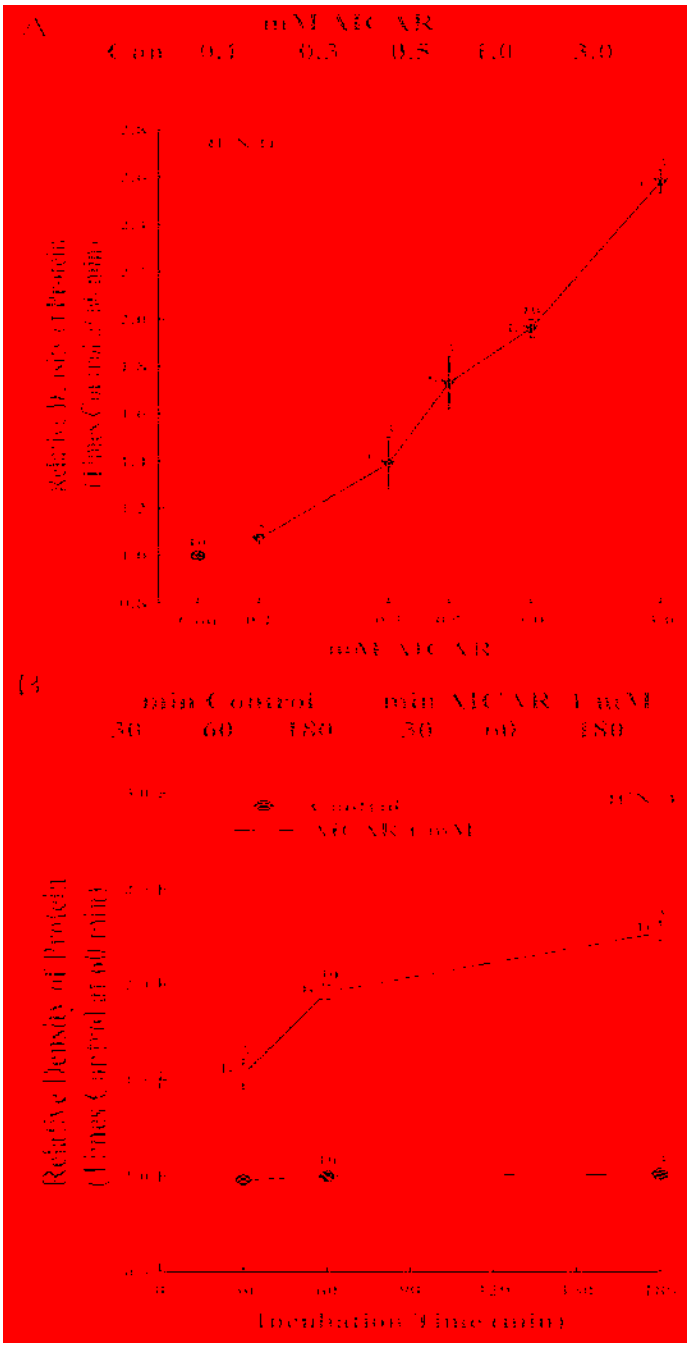

Figure 5-9: The dose-dependent and time-dependent effects of AICAR on the expression of JUN D in the ZF.

The expression of JUN D was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN D bands divided by the absorbance of the control JUN D bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

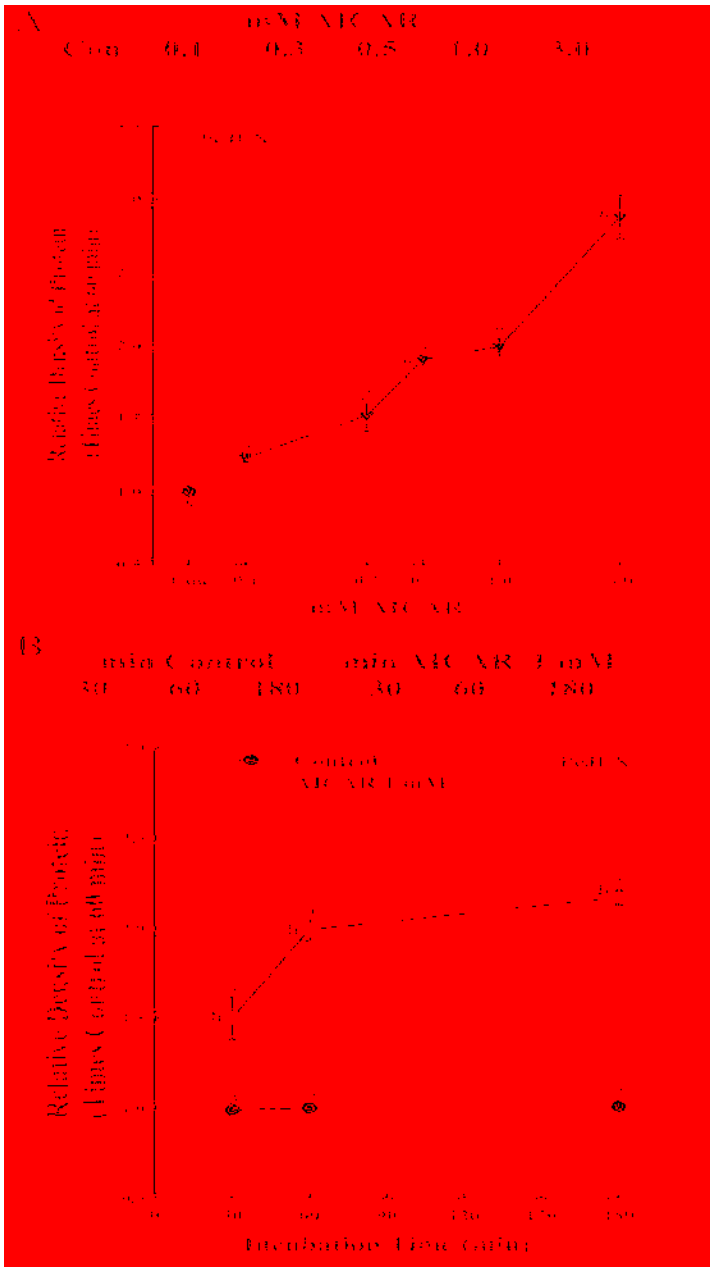

Figure 5-10: The dose-dependent and time-dependent effects of AICAR on the phosphorylation cJUN in the ZF.

Phosphorylated cJUN (PcJUN) content was determined utilizing western blots and the values expressed as a ratio of the absorbance of the treatment PcJUN bands divided by the absorbance of the control PcJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZF tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

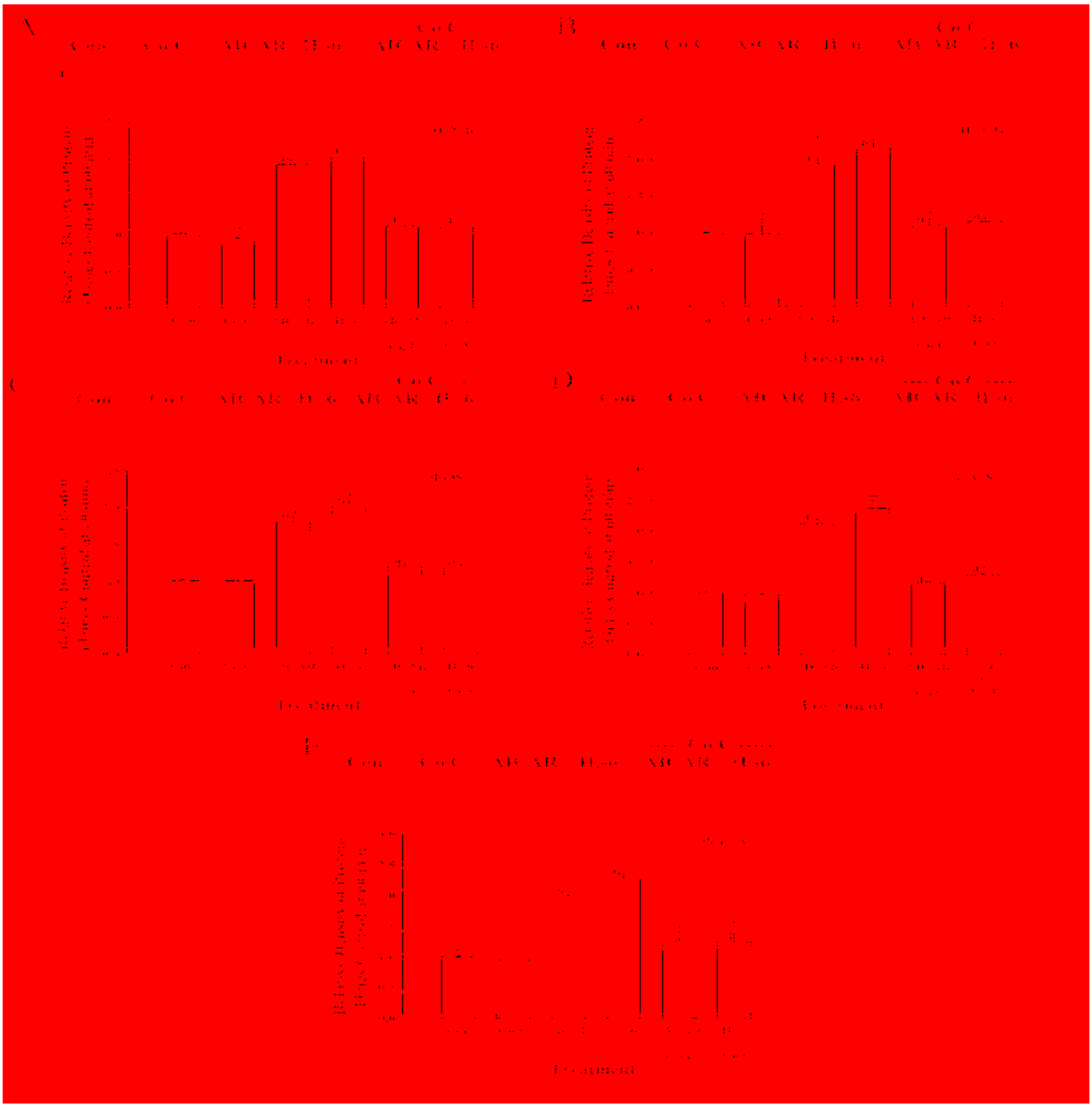

Figure 5-11: The effects of compound C on the expression of AP-1 subunits cFOS, cJUN, JUN B, JUN D, and on the phosphorylation of cJUN (PcJUN) in the ZF.

Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium (Control) or 10 µM compound C. The incubation medium was then removed and the ZF fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR+10 µM compound C; 25 ng/ml IL-6+10 µM compound C. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated a representative western blot for (A) JUN B; (B) JUN D; (C) cFOS; (D) cJUN; (E) PcJUN. In the lower section of each panel is a graph of the combined results from multiple experiments with the bars representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar. (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6)).

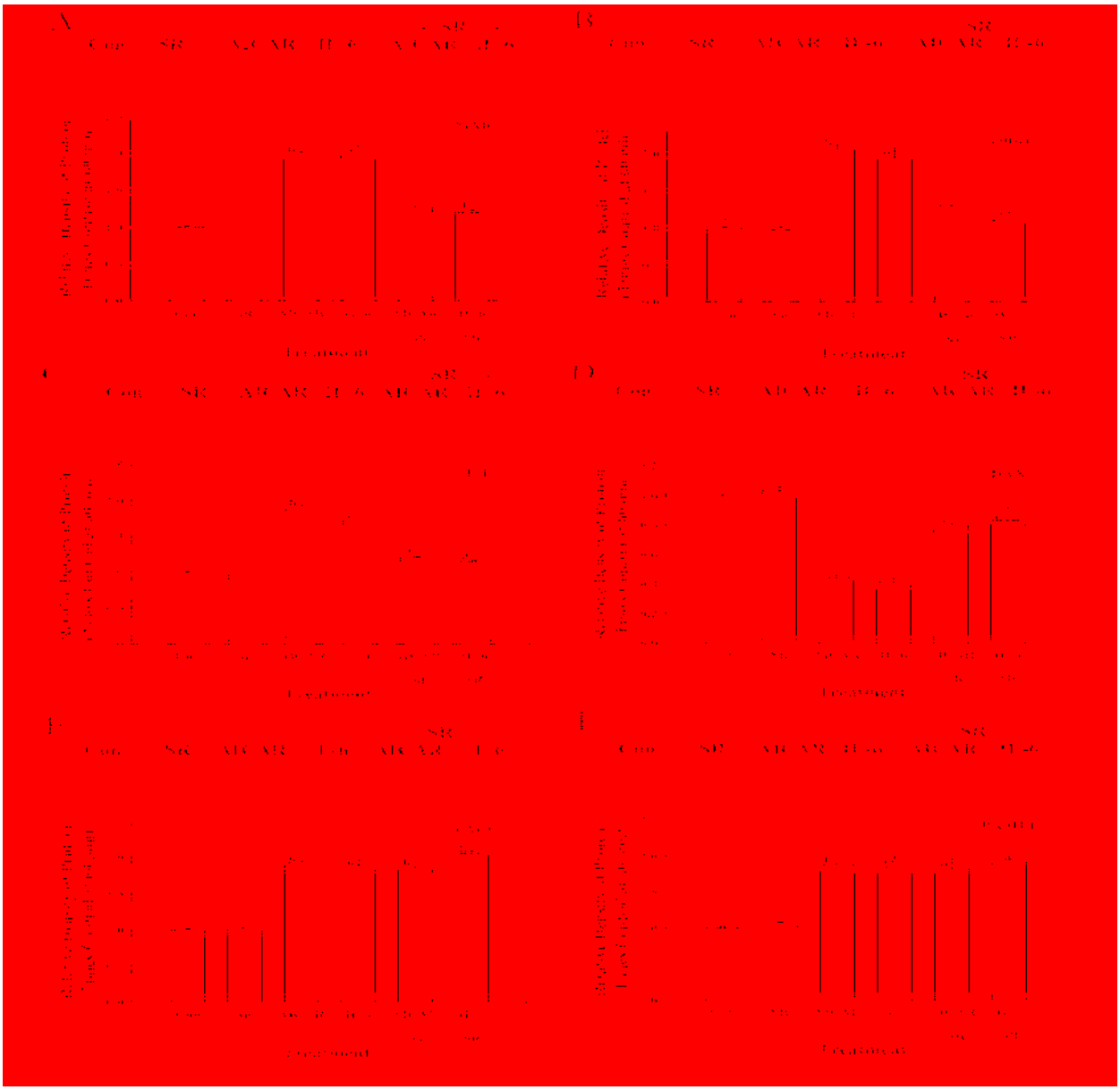

Figure 5-12: The effects of SR11302 on basal, IL-6-stimulated, and AICAR-stimulated expression of steroidogenic factors, PACC, and PAMPK in the ZF.

Adrenal ZF tissue fragments were incubated for 60 minutes with RPMI medium (Control) or 1.0 µM SR11302. The incubation medium was then removed and the ZF fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR+1.0 µM SR11302; 25 ng/ml IL-6 +1.0 µM SR311302. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated a representative western blot for (A) StAR; (B) P450scc; (C) SF-1; (D) DAX-1; (E) PACC; (F) PAMPK. In the lower section of each panel is a graph of the combined results from multiple experiments with the bars representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6).

CHAPTER 6: THE ACTIVATOR PROTEIN-1 COMPLEX FUNCTIONS AS A BIOCHEMICAL INTERMEDIATE IN THE INTERLEUKIN-6 AND AMP-ACTIVATED PROTEIN KINASE REGULATION OF STEROIDOGENIC ENZYMES IN THE ZONA RETICULARIS

Introduction

Previous research performed in our laboratory demonstrated that interleukin-6 (IL-6) decreases adrenal androgen release from the adrenal zona reticularis (ZR) [14, 16]. Furthermore in clinical studies patients suffering from chronic inflammatory stress exhibited increased plasma concentrations of IL-6 and decreased plasma concentrations of adrenal androgens [22]. Therefore, it was hypothesized that increased plasma IL-6 may lead to adrenal androgen deficiency. In support of this hypothesis, during aging the plasma concentration of IL-6 increases in mice, rats, monkeys, and humans, while adrenal androgens decrease. In male humans, the plasma IL-6 concentrations negatively correlate with the plasma concentration of DHEAS [8]. Furthermore, neutralization of plasma IL-6 with antibodies increases the adrenal androgen plasma concentrations in humans with rheumatoid arthritis [22].

IL-6 activates AMP-dependent protein kinase (AMPK), and this activation appears to be necessary for the IL-6 inhibition of expression of the steroidogenic proteins, steroidogenic acute regulator protein (StAR) and cholesterol side chain cleavage enzyme (P450scc) (Chapter 4). Additionally the IL-6-induced activation of AMPK decreases the expression of the nuclear factor steroidogenic factor-1 (SF-1), and increases the expression of the nuclear factor dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-

1) in the ZR (Chapter 4). Moreover, AMPK activates the activator protein-1 (AP-1) nuclear factor complex in AtT20 corticotrophs, a pituitary tumor cell line [59].

Similar to the adrenal zona fasciculata (ZF), various subunits of AP-1 such as cFOS, cJUN, JUN B, and JUN D are expressed in the adrenal ZR [16]. Furthermore, AP-1 regulates steroidogenesis in the ovary, testes, and adrenal ZF [56, 57, 58, Chapter 5]. Therefore, it is probable that AP-1 plays a role in regulating steroidogenesis of adrenal androgens in the ZR. Since AP-1 mediates the IL-6-induced changes in steroidogenic factors in the ZF (Chapter 5), it is likely that AP-1 may function as a nuclear transcription factor to inhibit the expression of steroidogenic proteins in the ZR. In support of this inhibitory role of AP-1 in the ZR, in steroidogenic tissues AP-1 can either stimulate or inhibit the expression of StAR depending on the subunit composition of the AP-1 complex [74]. Thus, it was hypothesized that IL-6 regulates AP-1 through the activation of AMPK, and AP-1 in turn inhibits the expression of StAR and P450scc in the ZR.

In order to test this hypothesis, the effects of IL-6 and the AMPK activator 5-amino-imidazole-4-carboxamide riboside (AICAR) on AP-1 protein subunit expression in adrenal ZR tissue were determined. Furthermore, the relationship between AMPK activation and AP-1 expression was investigated by determining the effects of the AMPK inhibitor compound C on IL-6 and AICAR-induced changes in AP-1 subunit expression in ZR tissue. The relationship between AP-1 subunit expression and the expression of steroidogenic proteins in the ZR was determined by utilizing the AP-1 inhibitor SR11302.

Results

IL-6 Regulation of AP-1 in the ZR

The relationship between AP-1 and IL-6 in regulating steroidogenic enzymes in the ZR was investigated by determining the effects of IL-6 on the expression of AP-1 subunits. IL-6 dose-response and time-course experiments were performed on adrenal ZR tissue fragments as described in Materials and Methods, and the expression of the AP-1 subunits cFOS, cJUB, JUN B, and JUN D was determined by western blots. The cytokine IL-6 significantly increased the expression of cFOS (Fig. 6-1-A), cJUB (Fig. 6-2-A), JUN B (Fig. 6-3-A), and JUN D (Fig. 6-4-A) in a dose-dependent manner. The expression of these AP-1 subunits were also dependent on the IL-6 exposure period in that the expression of cFOS (Fig. 6-1-B), cJUN (Fig. 6-2-B), JUN B (Fig. 6-3-B), and JUN D (Fig. 6-4-B) also increased in a time-dependent manner.

Phosphorylation of cJUN enhances the activation of the AP-1 transcription complex [55], and therefore the effect of IL-6 on cJUN phosphorylation was determined. The phosphorylation of cJUN significantly increased in a dose-dependent (Fig. 6-5-A) and time-dependent (Fig. 6-5-B) manner when the ZR tissue fragments were exposed to IL-6. Thus, IL-6 increased phosphorylation of cJUN as well as it increased the expression of the AP-1 subunits in the ZR tissue.

Effects of AMPK Activation on AP-1 in the ZR

The cytokine IL-6 increases the activation of AMPK in ZR tissue (Chapter 4). Furthermore, IL-6 increases the expression of AP-1 subunits and the phosphorylation of cJUN in the adrenal ZF (Chapter 5). Therefore, the effects of the AMPK activator AICAR on the expression of AP-1 subunits and cJUN phosphorylation were determined in the ZR. AICAR dose-response and time-course experiments were performed on adrenal ZR tissue fragments and

the expression of AP-1 subunits and PcJUN was determined by western blotting. The expression of cFOS (Fig. 6-6-A), cJUN (Fig. 6-7-A), JUN B (Fig. 6-8-A), JUN D (Fig. 6-9-A) and PcJUN (Fig. 6-10) significantly increased in a concentration-dependent manner in response to increasing concentrations of AICAR. Similarly, IL-6 significantly increased the expression of cFOS (Fig. 6-6-B), cJUN (Fig. 6-7-B), JUN B (Fig. 6-8-B), JUN D (Fig. 6-9-B) and PcJUN (Fig. 6-10B) in a time-dependent manner. Thus AMPK activation increased the phosphorylation of cJUN and increased the expression of the AP-1 subunits in the ZR tissue.

Effects of AMPK Inhibition on AP-1 in the ZR

The above results support a role for AMPK activation in the AICAR-induced and IL-6-induced expression of the AP-1 subunits and the phosphorylation of cJUN. In order to further test this hypothesis, the effects of the AMPK inhibitor compound C on IL-6-induced and AICAR-induced expression of AP-1 subunits and cJUN phosphorylation were determined. Compound C alone had no significant effect on expression levels of cFOS (Fig. 6-11-C), cJUN (Fig. 6-11-D), JUN B (Fig. 6-11-A), JUN D (Fig. 6-11-B) and PcJUN (Fig. 6-11-E) as compared to the control treatment (RPMI alone). Co-incubation with compound C and AICAR or IL-6 significantly decreased the expression of cFOS (Fig. 6-11-C), cJUN (Fig. 6-11-D), JUN B (Fig. 6-11-A), JUN D (Fig. 6-11-B) and PcJUN (Fig. 6-11-E) induced by AICAR or IL-6 in the ZR tissue. Thus, these results provide evidence that AMPK activation by AICAR or IL-6 mediated the increases in AP-1 subunit expression and phosphorylation of cJUN in the ZR tissue.

Effects of AP-1 Inhibition on Steroidogenic Factors and AMPK Activation in the ZR

AP-1 was functionally inhibited in a series of experiments using SR11302 in order to determine the role of AP-1 in regulating the adrenal steroidogenic factors StAR, P450scc, SF-1 and DAX-1 in the ZR. The adrenal ZR tissue fragments were exposed to SR11302 and western

blots were performed to quantify the expression of StAR, P450scc, SF-1, and DAX-1 in the ZR tissue fragments. SR11302 alone had no significant effect on the expression of StAR (Fig. 6-12-A), P450scc (Fig. 6-12-B), SF-1 (Fig. 6-12-C) and DAX-1 (Fig. 6-12-D), as compared to the control treatments (RPMI medium alone). Exposure of the ZR tissue to IL-6 or AICAR resulted in significantly decreased expression of StAR (Fig. 6-12-A), P450scc (Fig. 6-12-B), and SF-1 (Fig. 6-12-C), and significantly increased expression of DAX-1 (Fig. 6-12-D) as compared to the control treatments. The co-incubation of SR11302 with AICAR or IL-6 significantly attenuated the inhibition of expression of StAR (Fig. 6-12-A), P450scc (Fig. 6-12-B), and SF-1 (Fig. 6-12-C) mediated by AICAR and IL-6 treatments. In contrast, SR11302 blocked the increase of DAX-1 (Fig. 6-12-D) expression induced by AICAR or IL-6 treatments.

Furthermore in additional experiments, ZR tissue was co-incubated in SR11302 and AICAR or IL-6 to clarify the relationship between AP-1 and AMPK activation. It can be assumed that if AP-1 is downstream from AMPK activation in the biochemical pathway involved in AICAR and IL-6 modification of steroidogenic proteins, then SR11302 should not modify the IL-6 or AICAR activation of AMPK. The phosphorylation of AMPK (PAMPK) is an index of AMPK activation [68]. Activated AMPK phosphorylates the enzyme actyl CoA carboxylase (ACC) to form phosphorylated-ACC (PACC) and therefore PACC is also an index of AMPK activation [68]. SR11302 treatment had no significant effect on the AICAR or IL-6 induced increase of PACC (Fig. 6-12-E) and PAMPK (Fig. 6-12-F) in the ZR. Thus AP-1 did not affect AMPK activation in the ZR. In contrast, AMPK activation regulated AP-1 subunit expression and the phosphorylation of cJUN in the ZR.

Discussion

Although IL-6 increases the expression of AP-1 subunits in other tissues, the relationship between IL-6, AP-1, and the regulation of steroidogenesis had not been determined in steroidogenic tissues previously. Similar to the ZF (Chapter 5), IL-6 increases AP-1 subunit expression of cFOS, cJUN, JUN B, and JUN D, and the phosphorylation of cJUN in a concentration-dependent and time-dependent manner in the ZR. Therefore, after the adrenal ZF (Chapter 5), the adrenal ZR is the second steroidogenic tissue in which IL-6 has been demonstrated to increase AP-1 subunit expression and AP-1 phosphorylation.

Treatment with IL-6 activates the AMPK pathway in skeletal muscle, adrenal ZF (Chapter 3), and adrenal ZR (Chapter 4). AICAR stimulation of AMPK increases the activation of the AP-1 complex in a pituitary tumor cell line [59]. Furthermore, in the adrenal ZF, AMPK activation appears to be necessary for the IL-6-induced expression of the AP-1 subunits and the phosphorylation of cJUN (Chapter 5). Likewise, in this study AMPK activation by AICAR increases AP-1 subunit expression of cFOS, cJUN, JUN B, and JUN D, and increases the phosphorylation of cJUN in a concentration-dependent and time-dependent manner in the ZR tissue fragments. Compound C, an inhibitor of AMPK activation, blocks the increased expression of the AP-1 subunits and cJUN phosphorylation mediated by AICAR or IL-6 in the ZR. This observation provides evidence that the effects of AICAR and IL-6 on the AP-1 complex are mediated by AMPK activation. Furthermore, the compound C inhibition of the IL-6 effects on AP-1 supports a role of AMPK in the IL-6-induced expression of the AP-1 subunits and cJUN phosphorylation.

The AP-1 complex binds to the promoters of the genes for the steroidogenic enzymes StAR and P450scc. Depending on the subunit composition of the AP-1 complex and the

stimulation state of the cell, the AP-1 complex can either inhibit or stimulate the synthesis of mRNA for StAR and P450scc [73]. Therefore it is of interest that in the ZR AICAR or IL-6 inhibits the expression of the steroidogenic proteins StAR and P450scc. The nuclear factor SF-1 that stimulates steroidogenesis, and DAX-1 that inhibits steroidogenesis are likewise differentially regulated in the ZF and ZR, in that IL-6 and AICAR increase SF-1 and decrease DAX-1 in the ZF, but decrease SF-1 and increase DAX-1 in the ZR.

The AP-1 inhibitor SR11302 blocks the IL-6 and AICAR inhibition of StAR and P450scc expression in the ZR (Fig. 6-12). Therefore in the ZR, IL-6 appears to activate AMPK which in turn activates the expression of AP-1 subunits and cJUN phosphorylation. However, unlike in the ZF, the AP-1 complex in the ZR functions as an inhibitory complex that may bind to the promoters for the StAR and P450scc genes and inhibits the transcription of the mRNA for StAR and P450scc. This hypothesis is strengthened by the observation that SR11302 does not have a significant effect upon AMPK or ACC phosphorylation, and thus the effect of SR11302 cannot be explained by a non-specific inhibition of AMPK activation. IL-6 and AICAR decreased SF-1 and increased DAX-1 in the ZR, and these effects were blocked by the AP-1 inhibitor SR11302. Therefore, the IL-6 mediated changes in these nuclear factors also appear to be mediated through the AP-1 complex in the ZR.

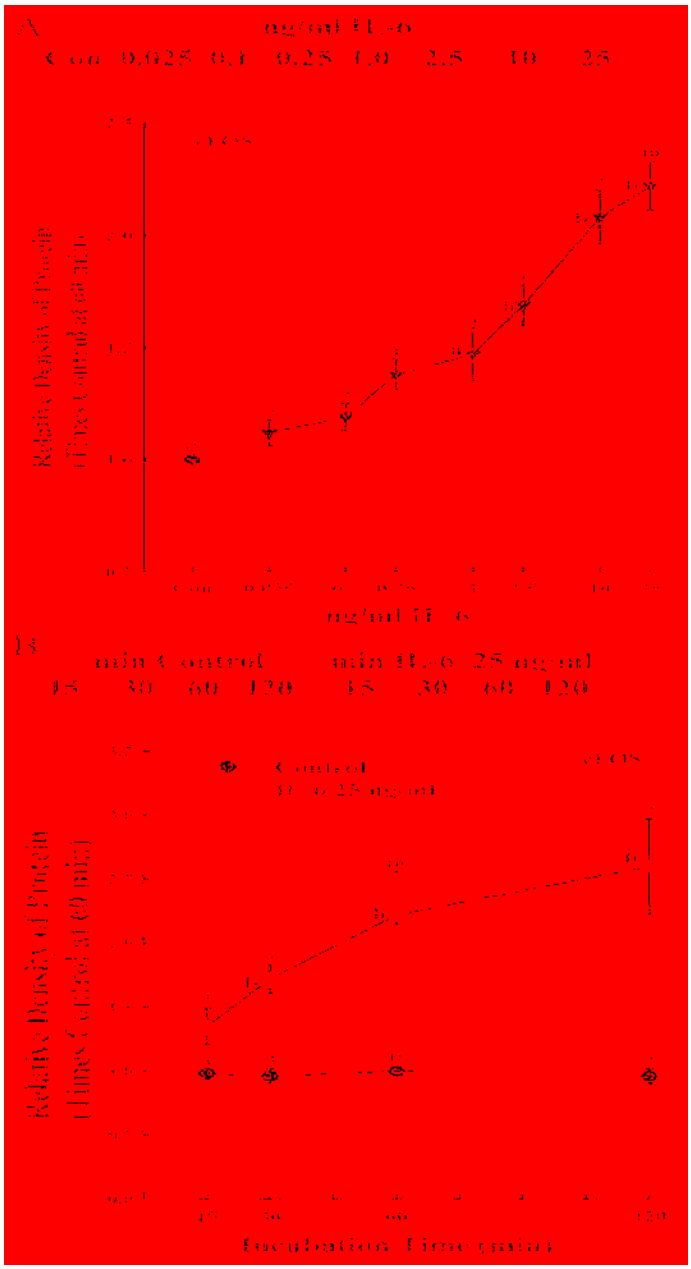

Figure 6-1: The dose-dependent and time-dependent effects of IL-6 on the expression of cFOS in the ZR

The expression of cFOS was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cFOS bands divided by the absorbance of the control cFOS bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

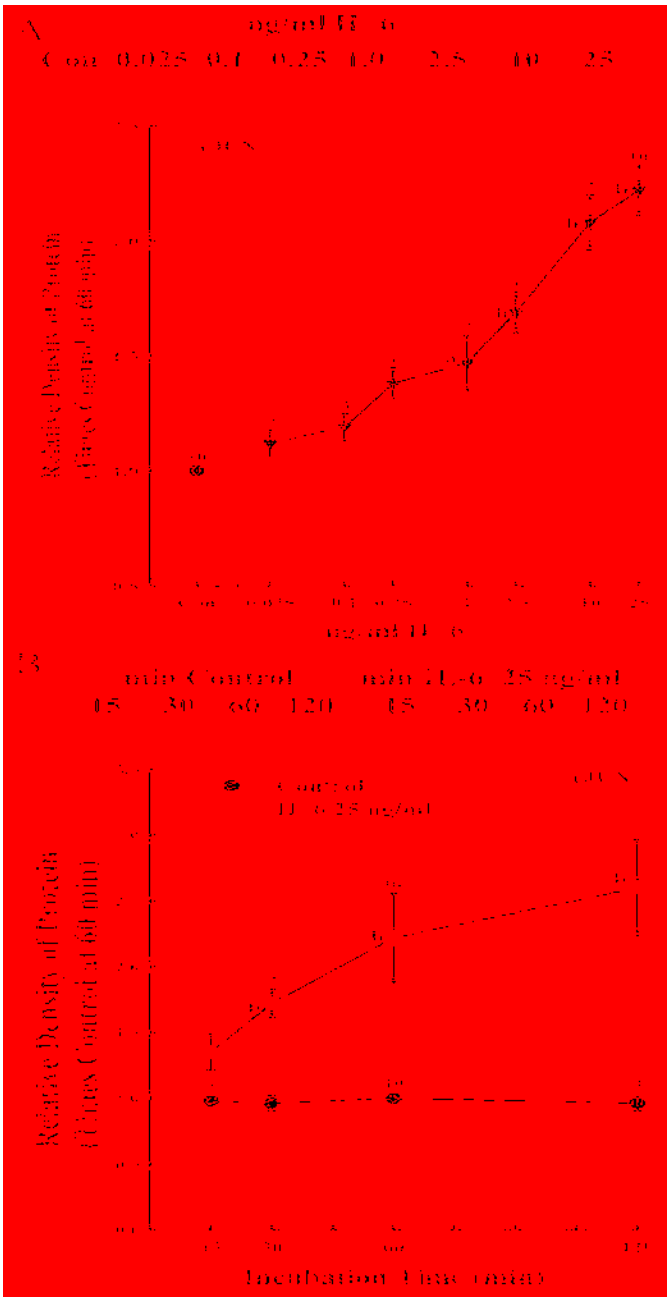

Figure 6-2: The dose-dependent and time-dependent effects of IL-6 on the expression of cJUN in the ZR

The expression of cJUN was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cJUN bands divided by the absorbance of the control cJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

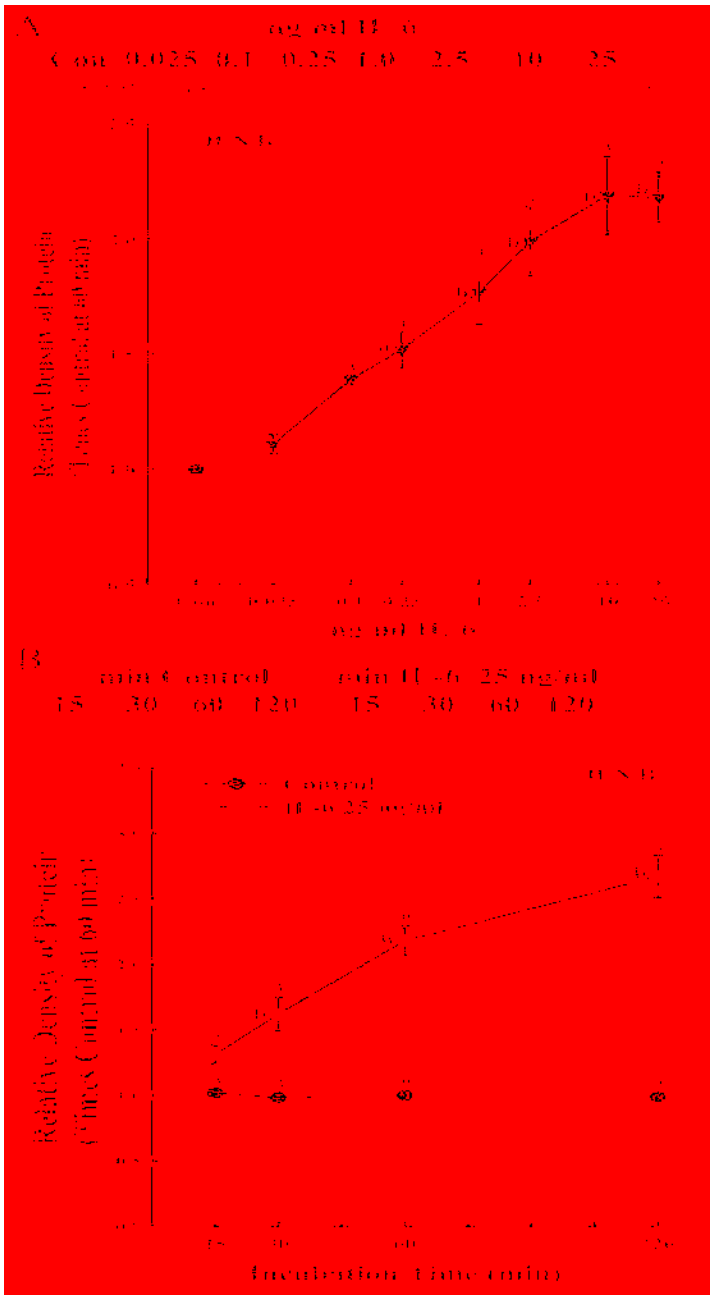

Figure 6-3: The dose-dependent and time-dependent effects of IL-6 on the expression of JUN B in the ZR

The expression of JUN B was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN B bands divided by the absorbance of the control JUN B bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

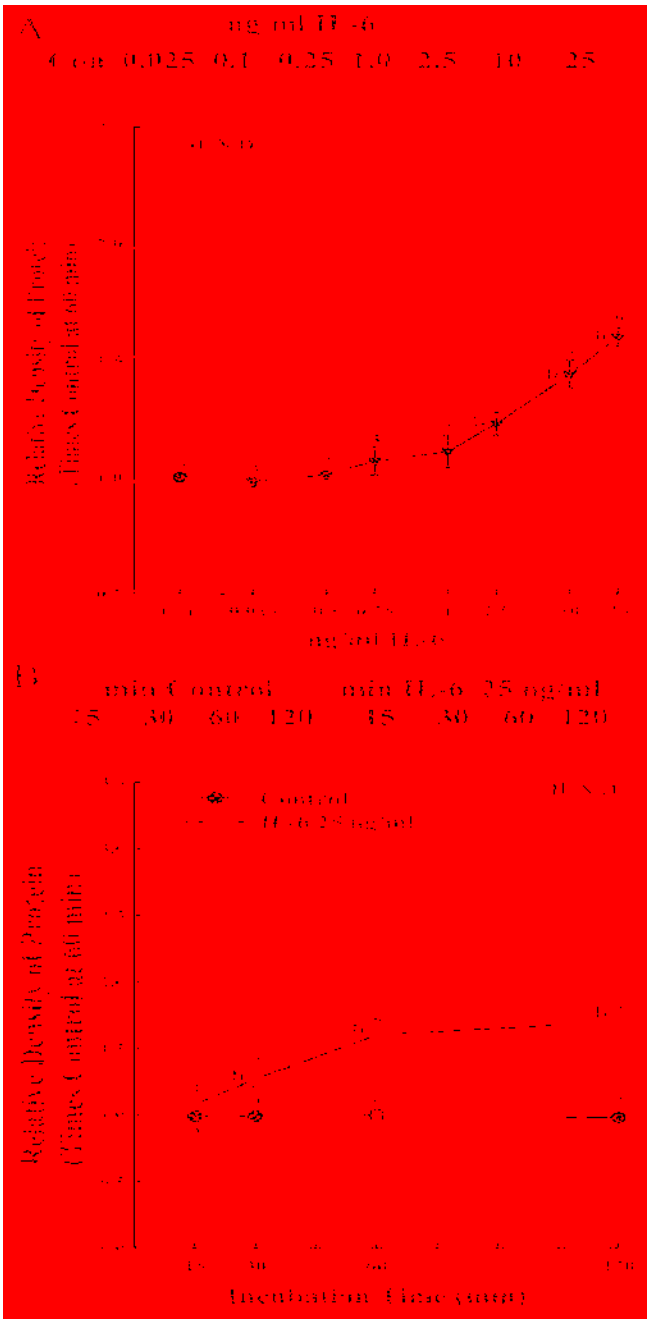

Figure 6-4: The dose-dependent and time-dependent effects of IL-6 on the expression of JUN D in the ZR

The expression of JUN D was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN D bands divided by the absorbance of the control JUN D bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

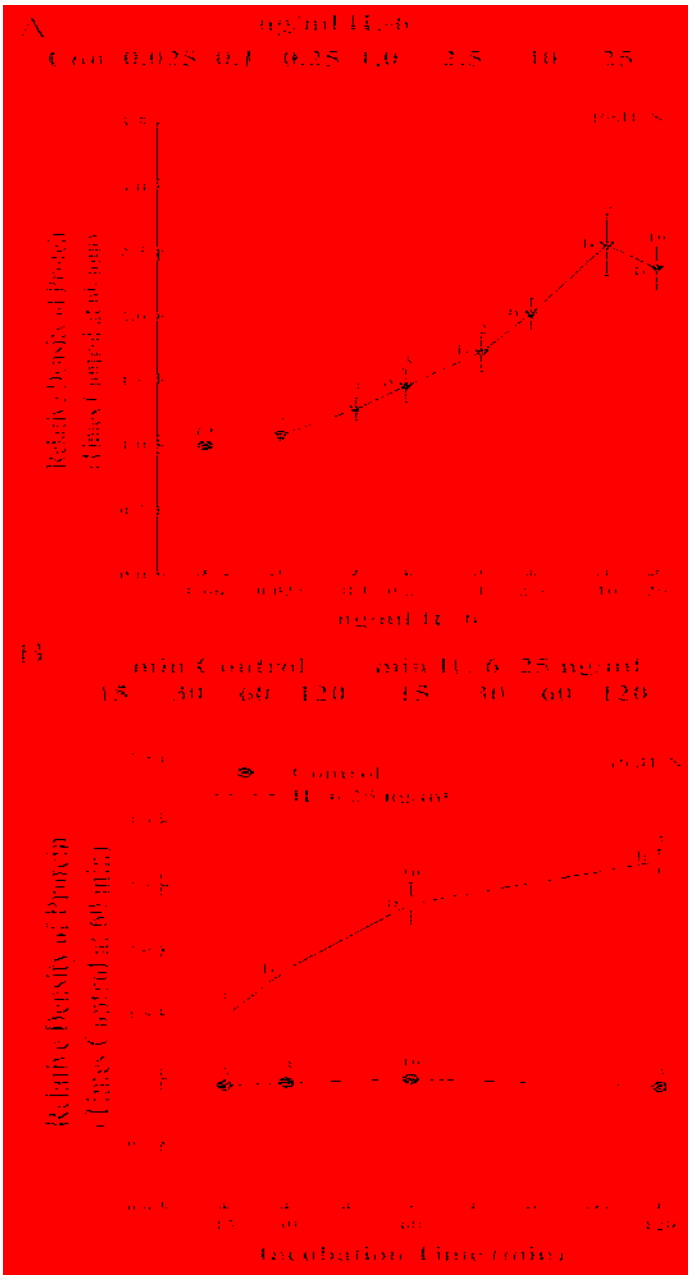

Figure 6-5: The dose-dependent and time-dependent effects of IL-6 on the phosphorylation of cJUN in the ZR

Phosphorylated cJUN (PcJUN) content was determined utilizing western blots and the values expressed as a ratio of the absorbance of the treatment PcJUN bands divided by the absorbance of the control PcJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 min with RPMI medium as the control (Con) or 0.025, 0.1, 0.25, 1.0, 2.5, 10 or 25 ng/ml IL-6. **Time- course experiment (B):** Adrenal ZR tissue fragments were incubated for 15, 30, 60, or 120 minutes with RPMI medium (Control) or 25 ng/ml IL-6.

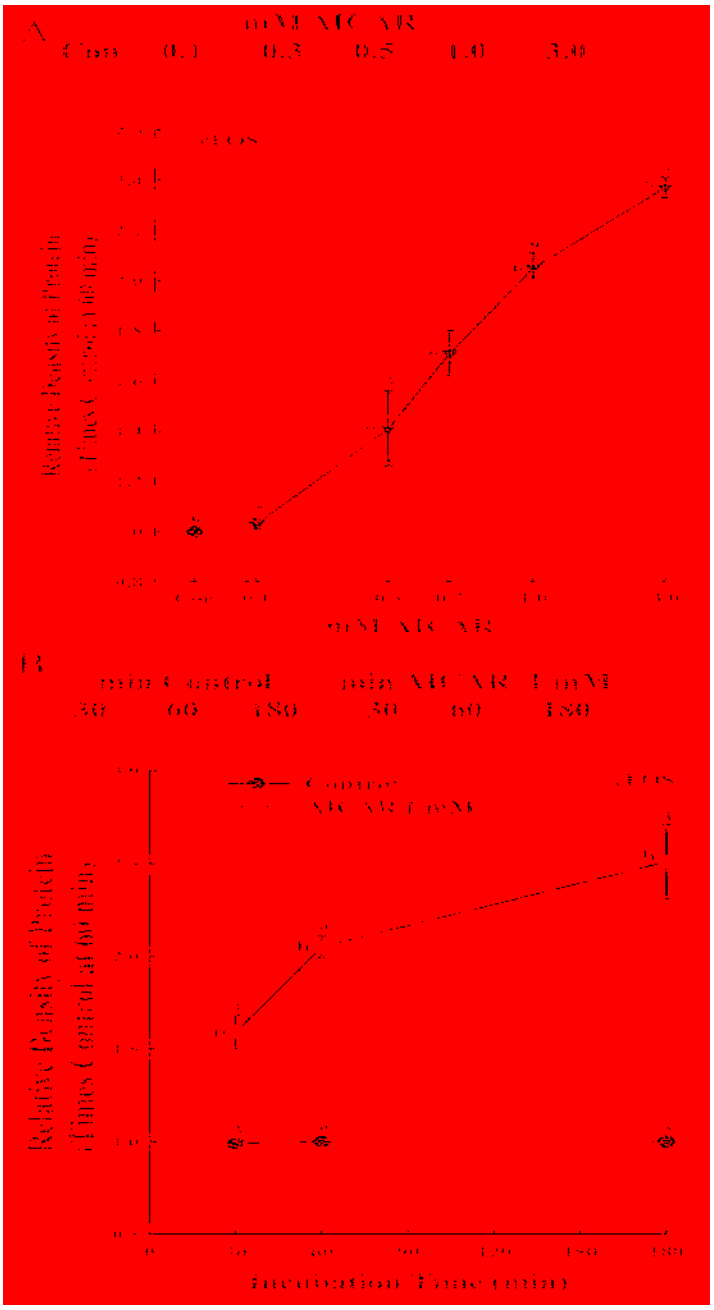

Figure 6-6: The dose-dependent and time-dependent effects of AICAR on the expression of cFOS in the ZR

The expression of cFOS was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cFOS bands divided by the absorbance of the control cFOS bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

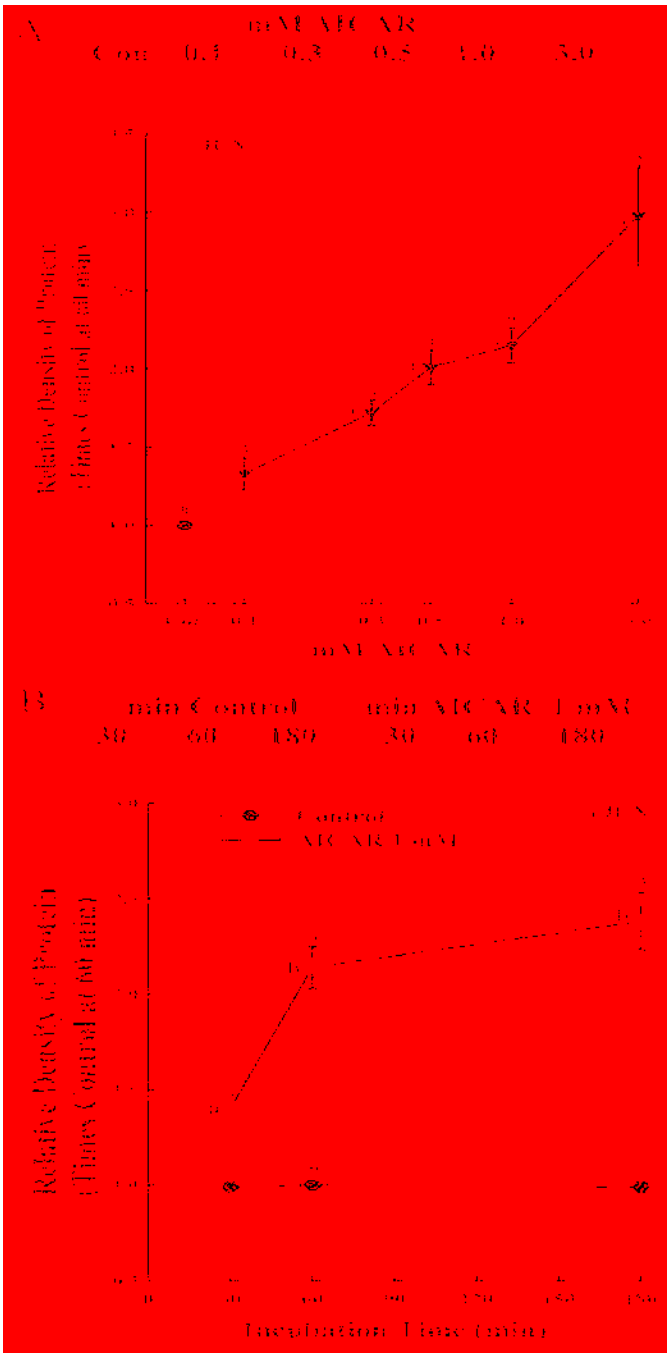

Figure 6-7: The dose-dependent and time-dependent effects of AICAR on the expression of cJUN in the ZR

The expression of cJUN was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment cJUN bands divided by the absorbance of the control cJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

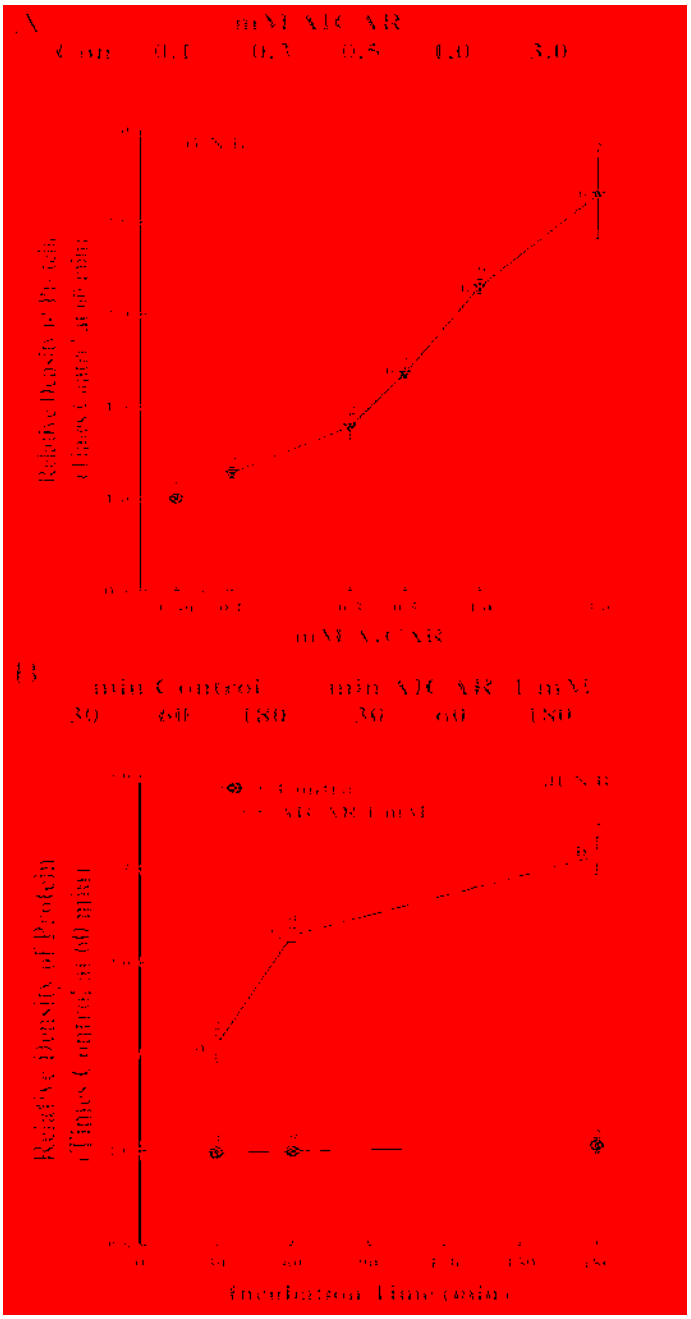

Figure 6-8: The dose-dependent and time-dependent effects of AICAR on the expression of JUN B in the ZR

The expression of JUN B was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN B bands divided by the absorbance of the control JUN B bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

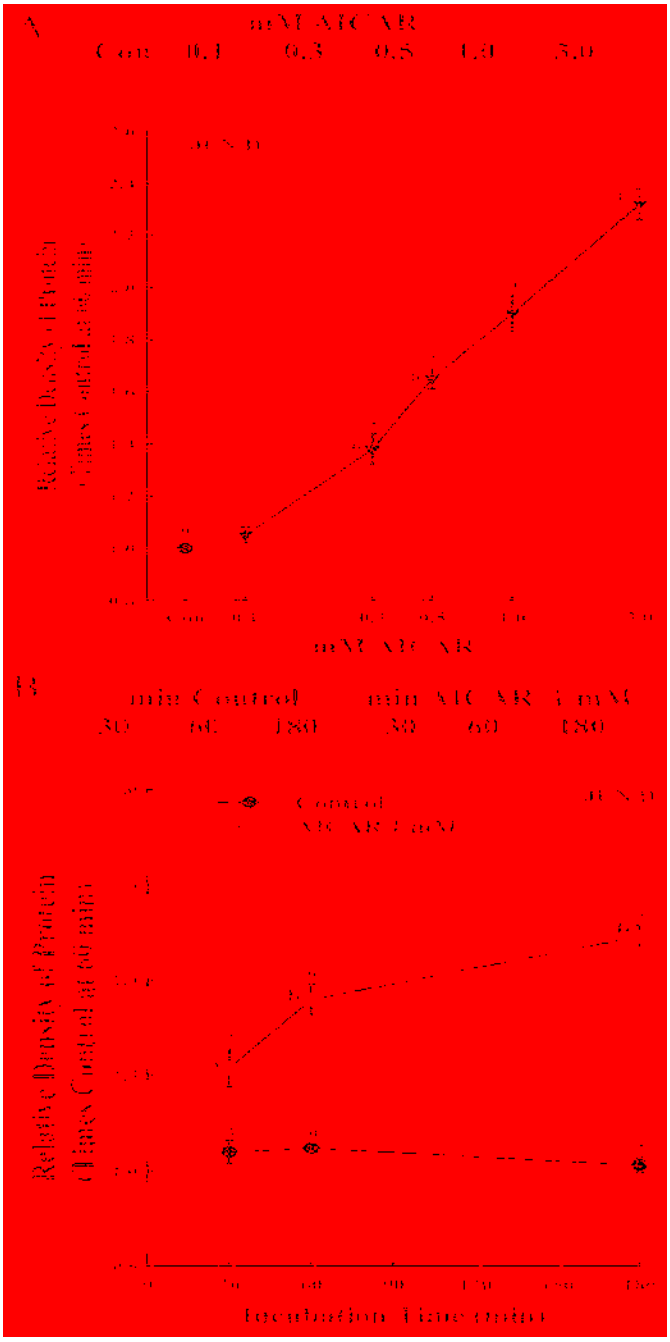

Figure 6-9: The dose-dependent and time-dependent effects of AICAR on the expression of JUN D in the ZR

The expression of JUN D was determined by western blotting and the values expressed as a ratio of the absorbance of the treatment JUN D bands divided by the absorbance of the control JUN D bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

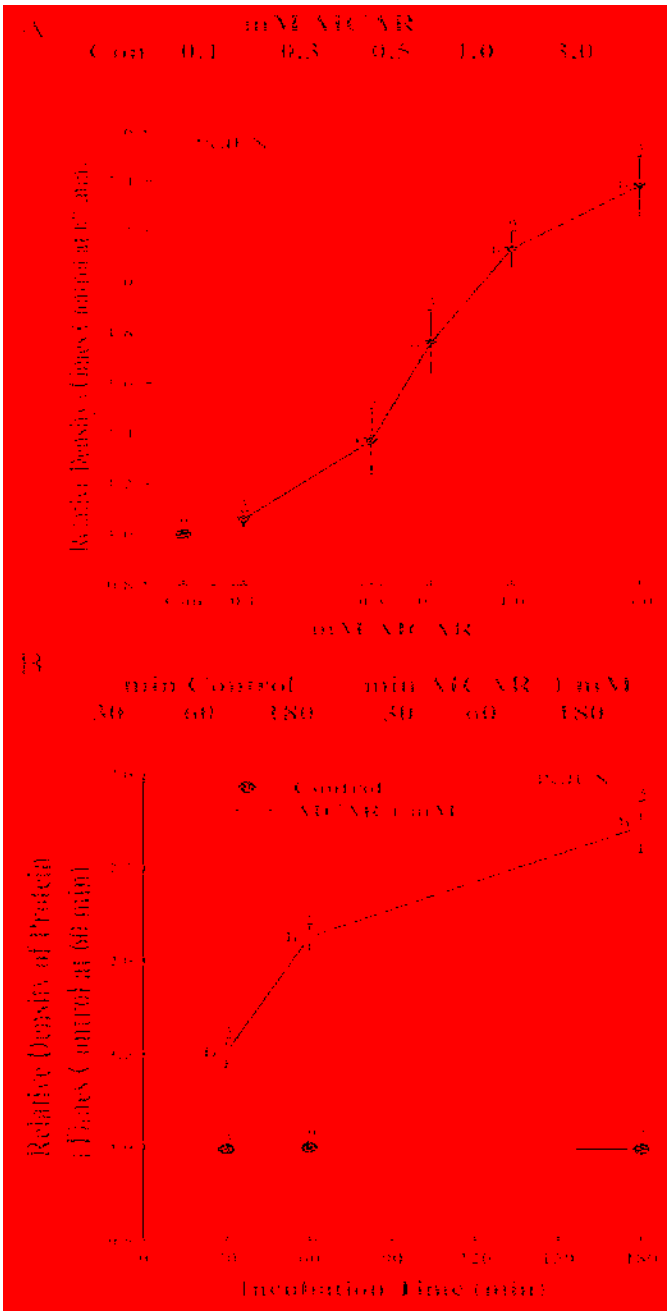

Figure 6-10: The dose-dependent and time-dependent effects of AICAR on the phosphorylation cJUN in the ZR

Phosphorylated cJUN (PcJUN) content was determined utilizing western blots and the values expressed as a ratio of the absorbance of the treatment PcJUN bands divided by the absorbance of the control PcJUN bands at 60 minutes. In the upper section of each panel is illustrated a representative western blot. The lower section of each panel is the combined results from multiple experiments with the points representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (a $p<0.05$ vs. control; b $p<0.01$ vs. control). **Dose-response experiment (A):** Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium as the control (Con) or 0.1, 0.3, .0.5, 1.0, or 3 mM AICAR. **Time-course experiment (B):** Adrenal ZR tissue fragments were incubated for 30, 60, or 180 minutes with RPMI medium (Control) or 1 mM AICAR.

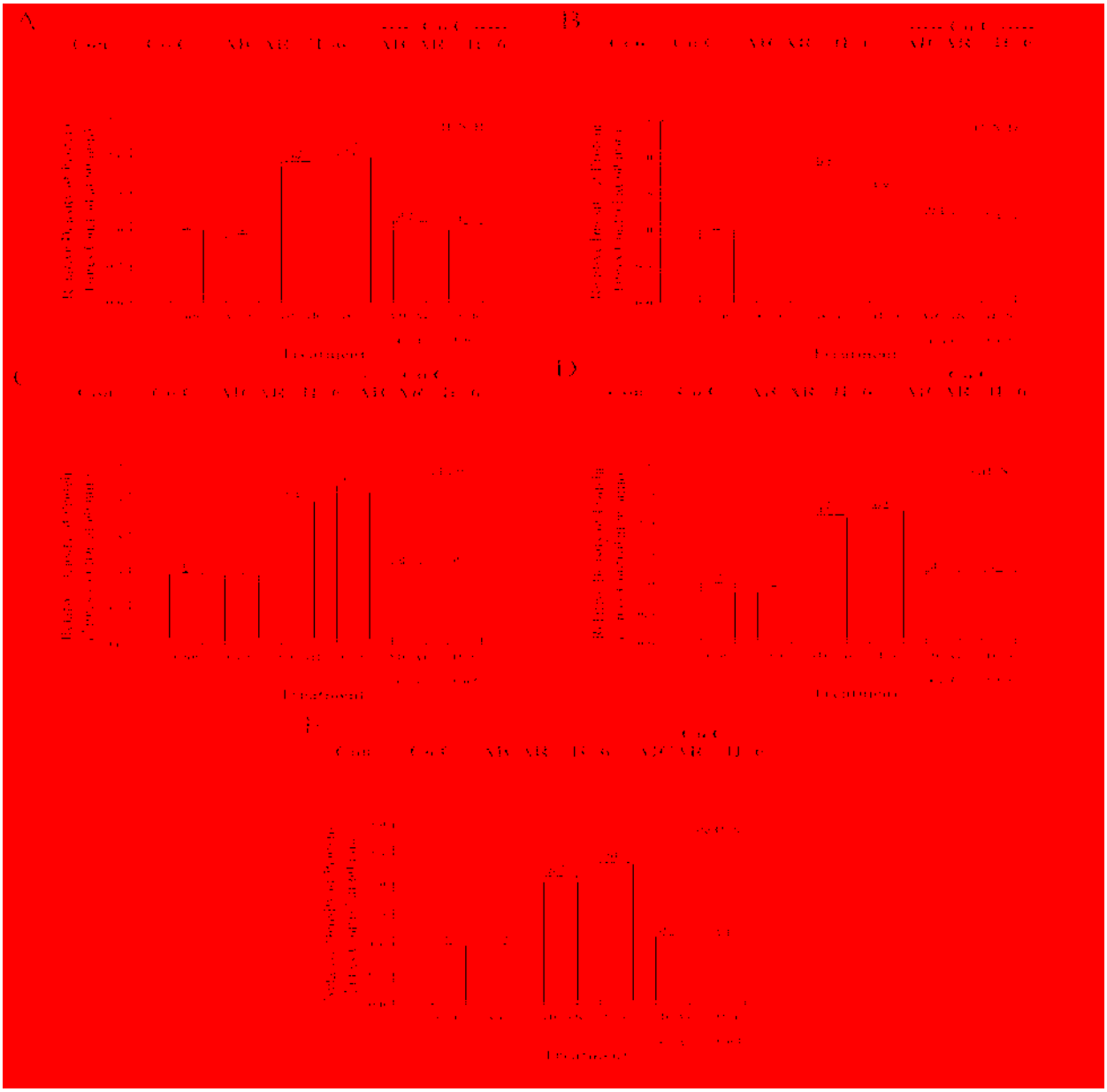

Figure 6-11: The effects of compound C on the expression of AP-1 subunits cFOS, cJUN, JUN B, JUN D, and on the phosphorylation of cJUN (PcJUN) in the ZR

Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium (Control) or 10 µM compound C. The incubation medium was then removed and the ZR fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR+10 µM compound C; 25 ng/ml IL-6+10 µM compound C. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated a representative western blot for (A) JUN B; (B) JUN D; (C) cFOS; (D) cJUN; (E) PcJUN. In the lower section of each panel is a graph of the combined results from multiple experiments with the bars representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar. (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6)).

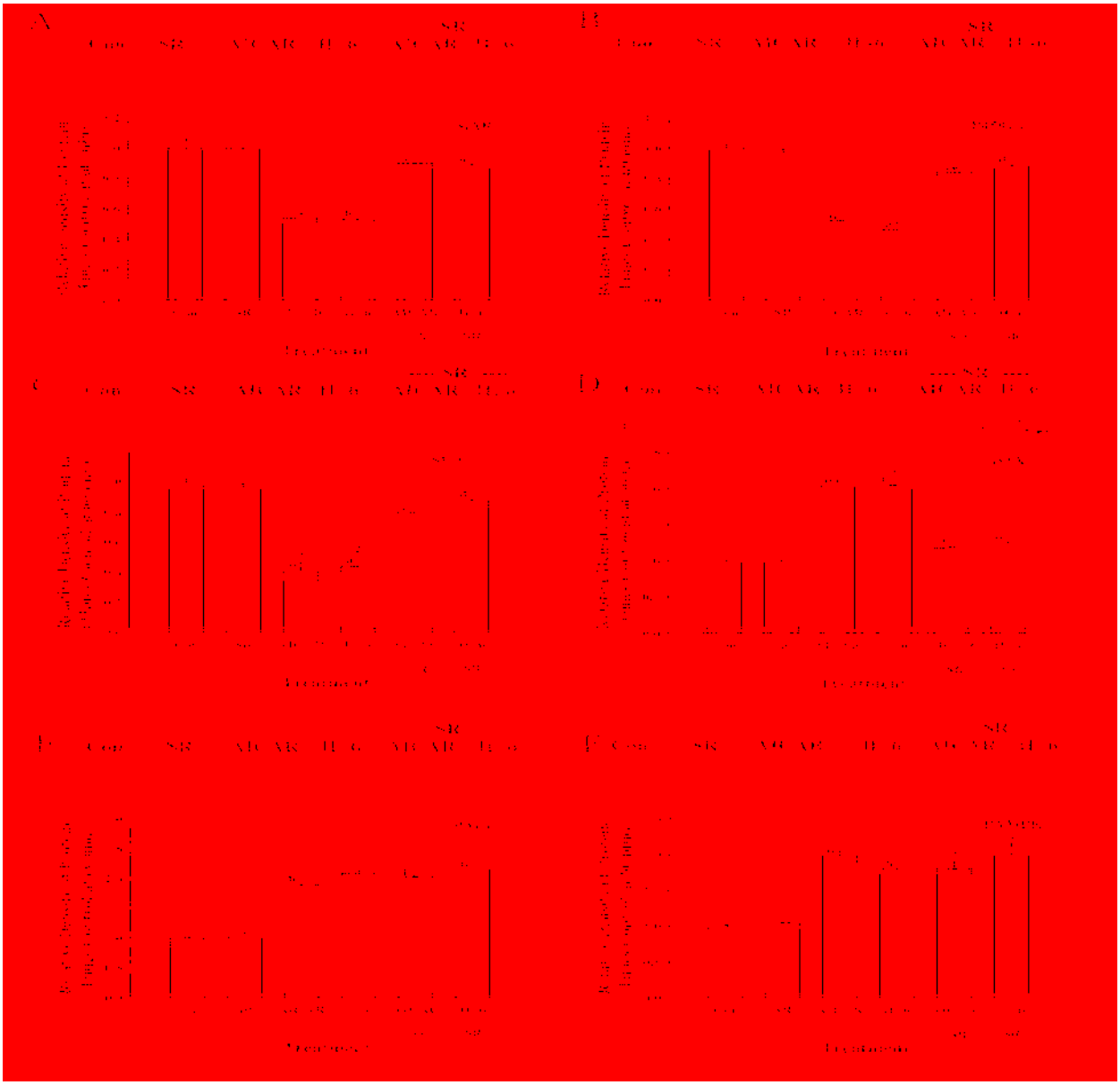

Figure 6-12: The effects of SR11302 on basal, IL-6-stimulated, and AICAR-stimulated expression of steroidogenic factors, PACC, and PAMPK in the ZR

Adrenal ZR tissue fragments were incubated for 60 minutes with RPMI medium (Control) or 1.0 μM SR11302. The incubation medium was then removed and the ZR fragments were exposed for 60 minutes to RPMI medium as control (Con); 1 mM AICAR; 25 ng/ml IL-6; 1 mM AICAR+1.0 μM SR11302; 25 ng/ml IL-6 +1.0 μM SR311302. The amount of the specific proteins expressed was determined by western blots and the values are expressed as a ratio of the absorbance of the protein band in the treatment group divided by the absorbance of the protein band in the control sample. In the upper section of each panel is illustrated a representative western blot for (A) StAR; (B) P450scc; (C) SF-1; (D) DAX-1; (E) PACC; (F) PAMPK. In the lower section of each panel is a graph of the combined results from multiple experiments with the bars representing the mean ± S.E.M. The number of different samples at each point is indicated by the numeral on top of each error bar, (b $p<0.01$ vs. control; d $p<0.01$ vs. corresponding treatment group (AICAR or IL-6)).

CHAPTER 7: SUMMARY

Discussion

Homeostasis plays an invaluable role in the survival of an organism. However, under pathological conditions the various reflexes that promote homeostasis may operate in a manner that becomes detrimental to the organism. Chronic stress is one such condition in which significant alterations in the balance of many physiological systems may be deleterious to the organism [1, 2]. In such times, the adrenal gland plays a major role in modulating the chronic stress response. The adrenal zona fasciculata (ZF) releases cortisol that increases blood glucose by increasing glucose release from the liver and decreasing the entry of glucose into skeletal muscle due to decreased insulin sensitivity. Cortisol also increases the catabolism of muscle proteins. Cortisol greatly inhibits the immune system and generally inhibits inflammation, although chronic exposure to high cortisol can promote inflammation of the central nervous system and the death of neurons in parts of the brain [3, 5]. The adrenal zona reticularis (ZR) releases various adrenal androgens. Many of the effects of adrenal androgens are opposite to those of cortisol in that adrenal androgens decrease blood glucose by potentiating the insulin sensitivity of skeletal muscle and promoting the synthesis of muscle proteins. Adrenal androgens enhance immune function and are protective of the central nervous system [1, 2].

During chronic inflammatory stress, ACTH returns to normal levels, but cortisol remains elevated. The increase in cortisol release has been hypothesized to be driven by the release of various cytokines including interleukin-6 (IL-6) [7]. In contrast to acute stress, chronic stress decreases adrenal androgens and this decrease may also be mediated by cytokines including IL-6 [1, 2, 7]. In the series of studies presented in this dissertation, I investigated the biochemical mechanisms involved in the IL-6-induced alterations of adrenal function, because a better

understanding of these biochemical pathways may lead to more effective treatment of chronic stress and aging.

The findings were consistent with the effects of IL-6 to stimulate cortisol release from the ZF, but inhibit adrenal androgen release from the ZR. The effects of AMPK on the ZF and ZR are particularly intriguing because AMPK is a major regulator of metabolism in many tissues and the organism as a whole [51]. The AMPK-mediated activation of steroidogenic pathways that may lead to cortisol release and the inhibition of pathways that may decrease adrenal androgen release are consistent with the role of AMPK in regulating overall metabolism [51].

In Figure 7-1 is illustrated the proposed pathways involved in the IL-6 regulation of StAR and P450scc in the adrenal ZF and ZR. There are however several important questions that remains unanswered by the research in this dissertation. Questions such as, how does IL-6 activate AMPK, how does AMPK activate the expression of the AP-1 subunits, and how does AP-1 modify SF-1 and DAX-1 expression when the promoters for these genes do not contain AP-1 binding elements, still remains to be answered.

Limitations

The most evident limitation of this study is that the steroidogenic pathway is analyzed in terms of steroidogenic enzymes only, and not by steroid hormone production. As mentioned earlier, the experimental model of ZF or ZR tissue fragments only supports the measurement of steroidogenic enzymes, and measuring steroid hormone synthesis and release would itself require dispersed ZF or ZR cell cultures. In spite of this limitation, it is still possible to safely assume that the changes in steroidogenic enzymes such as StAR and P450scc corresponds to changes in cortisol and adrenal androgens based on previous findings [25, 26, 27, 28, 29, 30].

The second most evident limitation is the direct effects of SF-1 and DAX-1 on StAR and P450scc. It is well established in the literature that SF-1 is a stimulatory transcription factor while DAX-1 is an inhibitory transcription factor involved in steroidogenesis [31, 32, 33, 35, 36, 37, 39, 41, 42, 43]. Although this particular study did not specifically look at how SF-1 and DAX-1 affects StAR and P450scc expression, the well-known fact that SF-1 is stimulatory while DAX-1 is inhibitory suggests that an increase in SF-1 and decrease in DAX-1 expression could be an underlying cause of increased expression of StAR and P450scc in the ZF; while the decrease in SF-1 and increase in DAX-1 could be an underlying cause of decreased StAR and P450scc in the ZR. Furthermore, previous studies have shown the presence of SF-1 and DAX-1 binding sites in the promoter regions of StAR and P450scc genes further supporting the idea of SF-1 and DAX-1 directly affecting StAR and P450scc expression at the transcriptional level [31, 32, 34, 37, 38, 40, 42].

AICAR has been well established as an AMPK activator. However it is possible to argue that AICAR may have non-specific and AMPK-independent effects on the ZF or ZR tissue thereby leading to the observed effects. Although the western blots showing that AICAR activates AMPK (Figs. 3-1, 4-1) prove that AICAR functions through AMPK activation in the ZF or ZR tissue, it is still possible that AICAR may also bring the observed changes in StAR and P450scc through other mechanisms. AMPK activation could be one of AICAR's several effects in the adrenal ZF or ZR tissue. Nevertheless Figs. 3-1 and 4-1 shows that these changes are at least partially AMPK dependent, and AMPK activation is at least partially responsible for the observed changes. Moreover the observation that compound C inhibits the AICAR effects strongly supports the hypothesis that the AICAR effects observed are primarily being mediated through AMPK activation (Figs 3-7, 3-8, 4-7, 4-8). A similar argument is also reasonable for

compound C and SR11302, where they could be exhibiting non-specific effects. Compound C and SR11302 have however been proven to be non-toxic to the ZF or ZR tissue fragments due to the fact that they increase protein expression in some of the treatments performed. Furthermore, SR11302 had no significant effect on AMPK activation in the ZF or ZR tissue (Figs. 5-12, 6-12).

Another limitation that should be addressed is the link between IL-6 and chronic stress. This particular study emphasizes the role of IL-6 as one of the key cytokines that regulate steroidogenesis in chronic stress. However it does not suggest that IL-6 is the only factor that regulates chronic stress. Other cytokines and hormones may also play a role in regulating steroidogenesis in chronic stress together with or independently of IL-6. For example, interleuken-4 and leukemia inhibitory factor, similar to IL-6 stimulates the release of cortisol from the ZF, but inhibits the release of androgens from the ZR [14, 15]. In this study, I determined the biochemical mechanisms through which IL-6 modifies adrenal function, because this cytokine is well established as a mediator of chronic stress effects on the adrenal gland.

Conclusions

IL-6 activates AMPK in both the adrenal ZF and ZR. Activation of AMPK in the ZF is responsible for the IL-6-induced stimulation of the steroidogenic peptides, steroidogenic acute regulatory peptide (StAR), and cholesterol side chain cleavage enzyme (P450scc). AMPK activation also increases the expression of the nuclear factor steroidogenic factor-1 (SF-1) that enhances steroidogenesis, and inhibits the expression of the nuclear factor dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1 (DAX-1) that suppresses steroidogenesis. Activation of AMPK in the ZR is responsible for the IL-6-induced inhibition of StAR and P450scc. AMPK activation also decreases the expression of SF-1 and stimulates the expression of DAX-1.

IL-6 increases the expression of the sub-units of activator protein -1 (AP-1) in both the ZF and ZR. AMPK activation increases the expression of activator protein-1 (AP-1) subunits in both the ZF and ZR, and AMPK activation is responsible for the IL-6 stimulation of the AP-1 subunit expression in the ZF and ZR. In the ZF, AP-1 increases the expression of StAR, P450scc, SF-1, and decreases the expression of DAX-1. In the ZR, AP-1 decreases the expression of StAR, P450scc, SF-1, and increases the expression of DAX-1. The contrasting effects of AP-1 in the ZF and ZR could be explained by the ability of the AP-1 transcription factor complex to dimerize in both stimulatory and inhibitory combinations.

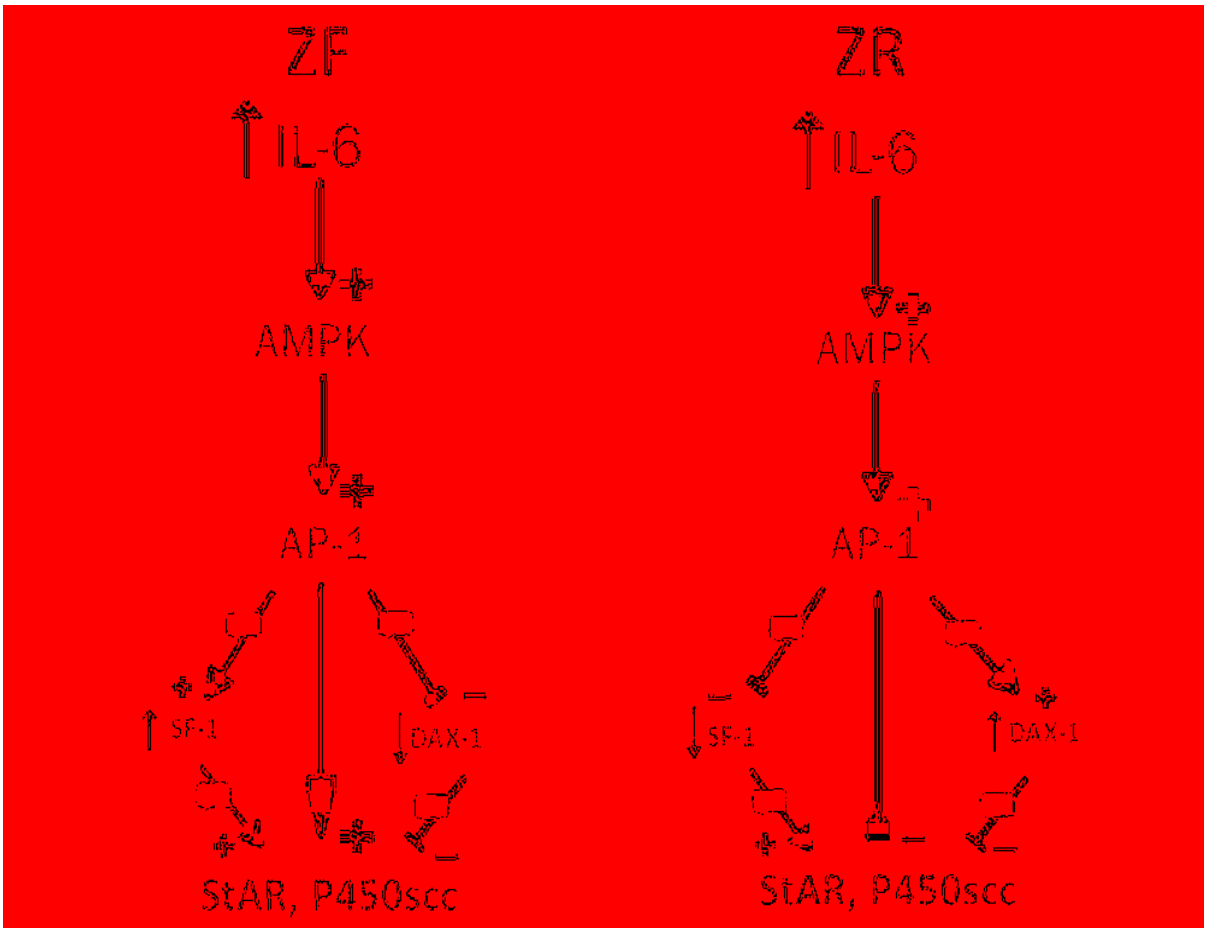

Figure 7-1: Proposed biochemical pathways through which IL-6 modifies the expression of StAR and P450scc in the ZF and ZR tissues

In the ZF, IL-6 activates AMPK which increases the expression of AP-1 subunits and the activation of AP-1 resulting in a stimulatory AP-1 complex. The stimulatory AP-1 complex increases the transcription of the mRNA for StAR and P450scc. The AP-1 complex increases the expression of SF-1 and decreases the expression of DAX-1 through alterations of intermediate regulators because neither DAX-1 nor SF-1 have AP-1 binding site in the respective promoters for the genes. The increase in SF-1 and decrease in DAX-1 augments the stimulatory effects of AP-1 on the StAR and P450scc promoters. Meanwhile in the ZR, IL-6 activates AMPK which increases the expression of AP-1 subunits and the activation of AP-1 resulting in an inhibitory AP-1 complex. The inhibitory AP-1 complex decreases the transcription of the mRNA for StAR and P450scc. AP-1 activation decreases the expression of SF-1 and increases the expression of DAX-1 through alterations of intermediate regulators. The decrease in SF-1 and increase in DAX-1 augments the inhibitory effects of AP-1 on the StAR and P450scc promoters.

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Academic Preparation

Ph.D. in Physiology & Developmental Biology 2013
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Dissertation: 'Involvement of AMPK and AP-1 Biochemical Pathways in IL-6 Regulation of Steroidogenic Enzymes in the Adrenal Cortex

B. S. in Physiology & Developmental Biology, 2010 2010
Brigham Young University, Provo Utah, USA

Professional Experience

Assistant Instructor – PDBio 365 Pathophysiology 2012
Lectured a class of 80 students on the concepts of Physiology and Pathophysiology; prepared presentations for the lectures.

Teaching Assistant – PDBio 365 - Pathophysiology 2011
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo, Utah, USA
Duties: Held help sessions and office hours to assist students in understanding basic concepts of Physiology and Pathophysiology.

Laboratory Instructor - PDBio 305 – Human Physiology Lab 2011-2012
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo, Utah, USA
Duties: Instructed 4 human physiology lab classes with 25 undergraduate students in each class; trained students in basic lab techniques used in medical settings; prepared and presented weekly lectures; graded laboratory reports and quizzes.

Graduate Research Assistant- Laboratory of Neuroimmunoendocrinology 2011-Present
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo Utah, USA
Duties: Design and carry out research projects on transcriptional & translational regulation of bovine adrenal steroiodogenesis; extract bovine adrenal glands at the abattoir; process bovine adrenal tissues for further experimentation; operate and maintain the laboratory equipment; modify and design experimental protocols.

Research Mentor - Laboratory of Neuroimmunoendocrinology 2011-Present
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo, Utah, USA
Duties: Mentor 20 undergraduate students working in the Laboratory of Neuroimmunoendocrinology

Awards

Academic Full Tuition Award 2011-2013
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo, Utah, USA

Research Assistantship 2011-2013
Dept. of Physiology & Developmental Biology,
Brigham Young University, Provo, Utah, USA

Dean's List 2010
Brigham Young University, Provo, Utah

Academic Full Tuition Scholarship 2008-2010
Brigham Young University, Provo, Utah

Academic Excellence Award - GCE Advanced Level 2007
Edexcel UK

'Summa cum laude' High School Graduation 2007
Asian International, Sri Lanka

Valedictorian 2007
Asian International School, Sri Lanka

Membership In Professional Organizations

The Endocrine Society 2012-Present

American Association for the Advancement of Science (AAAS) 2011-Present

Volunteer Service

Volunteer tutor at the Center for Service & Learning 2009-2010
Brigham Young University, Provo, Utah, USA

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