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HORMONAL AND STRESSOR-ASSOCIATED CHANGES IN PORCINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE ACTIVITY

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Summary—Cholesterol ester hydrolase (CEH) activity was characterized in the porcine adrenal gland and experiments conducted to determine the nature of its hormonal regulation. CEH activity was studied in the 14,000 g_{max} pellet (F4) and in the 192,000 g_{max} supernatant (F6). Characteristics associated with pH optima, product formation with time, linearity with increasing protein concentration, and equilibration of exogenous cholesterol esters added in acetone with endogenous cholesterol esters were determined. Scatchard analyses of saturation data demonstrated two-site models, which indicated the presence of lower velocity lower K_m enzymes (catalytic sites) (L-VK_m) and higher velocity higher K_m enzymes (catalytic sites) (H-VK_m) in both subcellular fractions. Neither ACTH (0.4 μg/kg body weight) nor 30-min restraint affected CEH activities at 0.5, 2, and 5 h after injection or initiation of restraint. However, 1 h after a longer restraint period (45 min), F4 H-VK_m CEH activity increased concomitantly with decreased F6 L-VK_m ($P = 0.003$). More modest increases in F4 H-VK_m ($P = 0.03$) were still apparent 1 h after the last of nine daily 45-min restraints. Bromocriptine (CB154, a dopamine agonist) administration for 6 days (9.6 mg/daily) reduced plasma prolactin (PRL) by 53% ($P < 0.05$), but had no effect on CEH activities. ACTH treatment to CB154-induced hypoprolactinemic barrows dramatically reduced F4 (63%) and F6 (49%) L-VK_m CEH activity ($P = 0.03$).

These data are the first concerned with regulation of adrenal CEH activity in swine, and are the first to evaluate *in vivo* treatments on *in vitro* CEH activity in any species evolutionarily higher than rodents. *In vivo* regulation of porcine adrenal CEH activity appears complex. Stressor-associated hormonal perturbations apparently must surpass a certain threshold of duration and/or magnitude before they alter CEH activity. Differing K_m and V_{max} of CEH within and between the two subcellular fractions studied and the differential responses to restraint stressor suggest that as many as four different enzymes with CEH activity are involved. Additionally, the combined effect of ACTH and CB154-induced hypoprolactinemia argues for an interrelated modulatory function of ACTH and PRL (or dopamine) on specific porcine adrenal CEH activities.

INTRODUCTION

Cholesterol is an acknowledged obligate precursor of steroidogenesis in adrenal tissue [1, 2]. Ultimately, most cholesterol used for adrenal steroidogenesis derives from blood-borne lipoproteins [3]. However, a more immediate source of cholesterol is cholesterol esters that are stored in membrane-bound lipid droplets [3–5]. These cholesterol esters are hydrolyzed into free cholesterol by a reaction which is catalyzed by the enzyme cholesterol ester hydrolase (CEH) [3, 6]. It was first demonstrated that ACTH produced a reduction in adrenal cholesterol [7], and subsequently proven that ACTH or stressors specifically depleted cholesterol esters [8]. These marked reductions in cholesterol esters were associated with increased CEH activity [9, 10].

The above-noted studies dealing with adrenal CEH and its *in vivo* regulation have been conducted with rats, and fewer studies have used guinea pig adrenals [11, 12]. Only one publication reported measurement of adrenal CEH in swine [13], and no information is available concerning its regulation in this species.

*Mention of names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Although ACTH is the pituitary-derived hormone most associated with activation of adrenal CEH, another proopiomelanocortin-associated peptide, increased CEH activity in the rat adrenal [14, 15]. Additionally, ACTH administration was unable to duplicate stressor-induced pituitary-dependent increases in adrenal CEH activity [16]. Such data suggest the involvement of a pituitary hormone other than ACTH in adrenal CEH activation. Prolactin (PRL) is a pituitary hormone which is also released in response to stressors in a variety of species including swine [17, 18]. It has a modulatory effect on adrenal function in adult rats [19], and in fetuses of other species [20, 21]. In pigs bromocriptine-induced hypoprolactinemia was associated with reductions in plasma cortisol [22], and adrenocortical PRL receptors have been measured in the porcine adrenal [23]. PRL has a known stimulatory effect on rat luteal CEH activity [24], but was without effect on rat adrenal CEH [10]. Therefore, the current studies characterized porcine adrenocortical CEH activity and determined effects of restraint stressor, ACTH, and bromocriptine-induced hypoprolactinemia on porcine adrenal CEH activity.

EXPERIMENTAL

Animals, surgery, blood and tissue collections

All pigs used were crossbred ($\frac{1}{4}$ Yorkshire: $\frac{1}{4}$ Landrace: $\frac{1}{4}$ Large White: $\frac{1}{4}$ Chester White) castrated male pigs (barrows) that were bred and raised in the swine facilities of the Roman L. Hruska U.S. Meat Animal Research Center (MARC). Tissue used for characterization of adrenal CEH activity was obtained from barrows slaughtered in the MARC abattoir by approved USDA procedures. For Experiment I, 60 pigs with an average weight at slaughter of 54.7 ± 0.4 kg were housed in an indoor facility in individual pens measuring 1.2×0.6 m, and had visual, olfactory, auditory, and tactile contact with another animal in an adjacent pen. These pigs were maintained at 20 ± 5°C. For Experiment II, 12 pigs with an average weight at the beginning of the study of 34.3 ± 0.9 kg were again housed as were pigs in Experiment I. For Experiment III, 20 pigs with an average weight at slaughter of 43.4 ± 1.9 kg were again housed in individual pens (1.22×1.22 m) with olfactory and auditory contact with other pigs in the same room and at a temperature of 22 ± 4°C. For all studies, pigs were fed a corn and soybean meal-based ration and water *ad libitum*, and were maintained at a photoperiod consisting of 12-h light (lights on 0600–1800 h).

Pigs were surgically catheterized in the external jugular vein and bled as described previously [22]. For sacrifice of experimental pigs, animals were anesthetized with sodium thiopental (Abbott Labs, North Chicago, IL, U.S.A.; 600–700 mg/animal) and immediately killed via exsanguination. Adrenal glands were removed and placed in 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl. Surrounding adipose tissue was dissected from adrenal glands, which were weighed, rapidly frozen in liquid nitrogen, and stored at −80°C.

Administration of bromocriptine and ACTH

Secretion of PRL was inhibited by use of the dopamine agonist bromocriptine mesylate (CB154; Innovative Research of America, Toledo, OH, U.S.A.). As described previously [22], pellets containing CB154 were implanted subcutaneously, and according to the manufacturer's specifications continuously secreted 9.6 mg CB154 per day. ACTH(1–24) (Cortrosyn, 25 U/250 μg; Organon, Oakforest, IL, U.S.A.) was injected through the catheter at a dosage of 0.4 μg/kg body weight. Pigs not receiving ACTH were administered a comparable volume of vehicle. Both vehicle and ACTH were flushed through the catheter with 10 ml of sterile heparinized saline [22].

Radioimmunoassay procedures

Double antibody radioimmunoassay (RIA) procedures were used for porcine PRL, porcine ACTH, and cortisol and have been described in detail previously [18, 22]. Plasma hormonal results for Experiment I have been reported [22]. For Experiments II and III, plasma cortisol measurements had a within-assay coefficient of variability (CV) of 2.8%, a between-assay CV of 12.2%, and a sensitivity based on the lowest concentration of cortisol in the linear range of the standard curve of 0.15 ng/tube. Plasma PRL measurements had a within-assay CV of 4.1%, a between-assay CV of 5.9%, and a sensitivity of 64 pg/tube. Plasma ACTH measurements had a within-assay CV of 3.4%, a between-assay CV of 12.2%, and a sensitivity of 1 pg/tube.

CEH assay

Tissue preparation. Adrenal glands previously stored at −80°C were thawed and the cortex

carefully dissected from the adrenal medulla. However, the existence of rays of chromaffin cells throughout the porcine adrenal cortex [25] precludes complete separation by dissection techniques. Dissected tissue was weighed and homogenized in 4 ml of ice-cold HEPES–sucrose solution (0.05 M HEPES, 0.25 M sucrose, pH 7.0) with a Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) for 1 min. The homogenate was filtered through the stainless steel support screen of a Gelman Easy-Pressure syringe filter holder (No. 4320, Gelman Sciences, Ann Arbor, MI, U.S.A.). This filtrate was centrifuged at $5000\,g_{max}$ for 10 min. The supernatant was removed from under the floating lipid layer and recentrifuged at $14{,}000\,g_{max}$ for 30 min. The supernatant was again removed from under the floating lipid layer and centrifuged at $192{,}000\,g_{max}$ for 1 h. The supernatant from this last centrifugation was used as the cytosolic source (F6) of CEH. The pellet from the $14{,}000\,g_{max}$ centrifugation was washed with 1 ml HEPES–sucrose, resuspended in 2 ml HEPES–sucrose, homogenized for 1 min, filtered, and used as the $14{,}000\,g_{max}$ source (F4) of CEH activity. All centrifugations were conducted at 2°C. Before each centrifugation, tissue preparations were overlaid with 30 μl of light mineral oil to reduce the extent to which the floating lipid layer broke up and contaminated supernatants in subsequent steps [26].

The CEH assay measures the conversion of cholesteryl oleate ([1-^{14}C]oleate) to [1-^{14}C]oleic acid and is similar to an assay previously described for CEH in adipose tissue [27]. Tissue preparations (50 μl $14{,}000\,g_{max}$ and 25 μl $192{,}000\,g_{max}$) were brought to a final volume of 500 μl with 0.05 M HEPES buffer containing 0.5% bovine serum albumin (BSA), pH 7.0 and were preincubated at 37°C for 2 min. Labeled (New England Nuclear, Boston, MA, U.S.A.) and unlabeled cholesteryl oleate (Sigma, St Louis, MO, U.S.A.) substrates solubilized in 10 μl acetone [23, 28] were added and incubations continued at 37°C for time intervals indicated in the text. Reactions were stopped by addition of 3 ml benzene–chloroform–methanol, 1:0.5:1.2 (by vol) that contained 0.1 mM unlabeled oleic acid (Sigma) as a carrier to increase extraction efficiency. [9,10-^{3}H]Oleic acid (20,000 dpm in 50 μl ethanol) was added to measure extraction efficiency. Subsequently, 400 μl of 0.75 N NaOH was added, the reaction mixture thoroughly mixed, placed at 4°C for 16–20 h, then centrifuged at $2000\,g_{av}$ for 20 min at 2°C. Subsequently, 1.6 ml of the upper water–methanol layer containing oleic acid was transferred to a scintillation vial, and ^{3}H and ^{14}C radioactivity counted using double-label counting procedures. Extraction efficiencies ranged from 75–90%. Nonspecific conversion of substrate to product was measured for each experimental sample. For this measurement, all ingredients were included, but reactions were stopped immediately after substrate addition.

Validation of CEH assay

Studies to determine effects of pH, product formation with time, and linearity of product formation with increasing concentrations of protein were conducted using conditions described in tables and figure legends. A study was conducted to ascertain the extent to which endogenous cholesterol esters would compete with labeled cholesterol oleate substrate added in acetone. "Pools" of heated (60°C for 10 min) F4 and F6 were prepared that served as a source of endogenous cholesterol esters within minimal CEH activity. Heating caused only a modest decrease in the levels of cholesterol esters (F4, 219 ± 37 μg/100 μl nonheated vs 213.3 ± 11.9 μg/100 μl heated; F6, 193 ± 5 μg/100 μl nonheated vs 174 ± 10 μg/100 μl in heated preparations). This test was conducted by incubating enzymatically active F4 and F6 preparations with labeled cholesteryl oleate (230,000 dpm; 3.6 μM) in the absence or presence of increasing quantities of exogenous or endogenous cholesterol esters. Exogenous cholesterol esters consisted of cholesterol oleate (0–80 μg) added in 10 μl acetone. Endogenous cholesterol esters consisted of heated F4 or F6 fractions (0–100 μl). All incubates were adjusted to a constant volume, incubated for 20 min at 37°C, and reactions stopped as described previously.

To measure saturating substrate concentrations and determine K_m and V_{max}, subcellular fractions (F4, 132–260 μg protein, $n = 4$; F6, $n = 3$, 220–380 μg protein) were prepared from adrenals of three different barrows. These preparations were incubated with increasing concentrations of labeled and unlabeled cholesteryl oleate (1–267 μM), but at a constant ratio of labeled to unlabeled substrate.

For each sample associated with Experiments I, II, and III, both the F4 and F6 fractions were prepared. CEH activity in each fraction was measured at substrate concentrations of 0.4 and 241 μM. These two concentrations were used to estimate activity of the low and high K_m/V_{max}

activities, respectively. CEH activity for F4 was measured using $65.7 \pm 3.9\ \mu g$ protein for a 20-min incubation period at 37°C. CEH activity for F6 was measured using $171.9 \pm 3.9\ \mu g$ protein for a 10-min incubation period at 37°C. One complete replicate for F6 was measured inadvertently at 20 min for Experiment I. Since all treatment groups were represented, this would not have adversely biased the data. All incubations were preceded by a 2-min preincubation period of the enzymatic preparations at 37°C. For Experiment I, 23 assays were conducted that had an average within-assay CV of 7.9% based on the triplicate determinatins for each sample for both F4 and F6 and at each substrate concentration. Between-assay repeatability was determined by measuring CEH activity at both high and low substrate concentrations in porcine pancreatic CEH (Sigma). The between-assay CV was 22.8%. For Experiment II, the within-assay CV was 9.1%, and the between-assay CV was 40% for the four assays conducted. This high latter CV apparently resulted from the more acidic pH. For Experiment III, the within-assay CV was 7.3% and the between-assay CV was 16.2% for 7 assays.

Cholesterol ester assay

Filtered homogenates from tissue preparations used for the CEH assay were assayed for concentrations of cholesterol esters using a modification of previously published densitometric procedures [29, 30]. Samples were extracted with chloroform–methanol (2:1, v/v) using cholesteryl oleate ([1-^{14}C]oleate) to measure extraction losses. After drying under nitrogen, a benzene resolubilized aliquot of extract was spotted on a thin layer chromatography plate (Analtech Preadsorbent Channeled Silica gel GHL TLC plate, 250 micron thickness, 20 × 20 cm, Newark, DE, U.S.A.), which was pretreated with 4% sulfuric acid. On each TLC plate for which there were experimental samples, a standard curve consisting of 1, 2, 3 and 4 μg of cholesteryl oleate was also spotted. TLC plates were developed in hexane–diethyl ether–acetic acid (75:21:1, by vol), then heated at 180°C for 20 min. Cholesterol esters were quantitated by use of a Shimadzu Densitometer (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.).

Accuracy and precision of this densitometric assay for adrenal cholesterol esters were determined. The average accuracy of estimates was 94%, and a plot of expected vs measured had a slope ($b = 0.80$) that did not differ from 1 ($P > 0.05$). The average CV for five standards, each assayed on three separate TLC plates was 4.1%. The average CV for the experimental samples (includes extraction, spotting, and chromatography on TLC plates, and densitometric analysis) was 8.5%.

Protein assay

Protein for CEH was measured in triplicate using a modification of the Lowry procedure [31].

Experimental design and procedures

Experiment I. As described previously [22], barrows were catheterized and implanted with CB154 (where applicable) on day 0, and were killed on day 6. Treatment groups included: control barrows ($n = 15$); barrows restrained for 30 min on day 6 ($n = 15$); barrows injected once with ACTH on day 6 ($n = 12$); barrows treated with CB154 ($n = 9$); and barrows treated with CB154 and ACTH ($n = 9$). Each of these five main treatment groups was divided further into barrows which were killed at 0.5, 2, or 5 h after injection of ACTH or vehicle or initiation of restraint. These time periods closely approximated 0830, 1000, and 1300 h. Restrained pigs were placed in a mobile, steel restraining cage with styrofoam padded sides to prevent physical injury to the animals. This cage held them in such a way that no lateral movement and only a very slight amount of forward–rearward movement was allowed. After the 30-min restraint, depending on the time of sacrifice, animals were killed immediately or returned to their pens until time of sacrifice.

Experiment II. There were two treatment groups with six barrows randomly assigned to each group: (1) control animals; and (2) barrows restrained for 30 min and killed immediately after this 30-min restraint period. Barrows were catheterized surgically 7–11 days prior to blood sample withdrawal. On the evening before blood sampling, a 1.5 m catheter extension was attached to the indwelling jugular catheter so that blood samples could be taken by individuals standing outside the animals' cages and without disturbing the animals. Basal blood samples were taken 10 min before restraint, animals were placed in a restraining device, and a second sample taken after the 30-min restraint, just before slaughter of the animal. Control barrows were sampled at similarly placed time intervals.

Experiment III. There were four treatment groups with five barrows randomly assigned to each group: (1) control animals; (2) barrows restrained 45-min daily for 9 days and then killed 1 h after the end of the restraint on day 9; (3) barrows restrained 45-min daily for 8 days and killed on day 9 approx. 24 h after the last restraint period; and (4) barrows restrained once on day 9 for 45 min and killed 1 h later. On day 9, blood samples were taken 20 min prior to restraint and at 3, 10, 20, 40, and 45 min after restraint initiation. Blood samples were taken at similar time intervals from control pigs and from those that had been treated for 8 days previously but were not restrained on day 9. In this study, the restraining device consisted of a mobile panel made of steel bars and padded with styrofoam, shaped to the contours of the pigs' bodies, and which could be placed inside the pigs' cages. Again, the device allowed pigs to stand or lay down and allowed some forward–rearward but no lateral movement.

All experimental procedures were approved by the USDA Meat Animal Research Center Committee for Care and Use of Domestic Animals and were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Statistical analyses

Enzymatic data were analyzed using two-way analysis of variance (ANOVA) for Experiment I (main factors consisted of treatment and time), and one-way ANOVA (Experiments II and III). Tests to determine significant differences among means used the Student–Newman–Keuls multiple range test and preplanned comparisons via partitioning treatment sums of squares and degrees of freedom (*df*) into single *df* comparisons [32]. Data were tested for normality of distribution and homogeneity of variance and were log or square-root transformed where necessary to fulfill assumptions of ANOVA [32]. For Experiment II, plasma hormonal concentrations were analyzed using repeated measures analysis [33]. Scatchard analyses were conducted using the computer program LIGAND [34].

RESULTS

Characterization of porcine adrenocortical CEH activity

Subcellular distribution. Activity was measured ($n = 3$) in the tissue homogenate (F1) and five subcellular fractions: $800\,g_{max} \times$ 10-min pellet (F2); $5000\,g_{max} \times$ 10-min pellet (F3); $14{,}000\,g_{max} \times$ 30-min pellet (F4); $192{,}000\,g_{max} \times$ 60-min pellet (F5); $192{,}000\,g_{max} \times$ 60-min supernatant (F6). The greatest specific activity (nmol/mg protein) occurred in the $14{,}000\,g_{max} \times$ 30-min pellet (F4) and represented an 11-fold increase in specific activity. Similarly, this subcellular fraction had approx. 40% of the total activity. The soluble cytosol ($192{,}000\,g_{max} \times$ 60-min supernatant; F6) had a 100% increase in specific activity and 15% of the total CEH activity (data not shown). Interpretation of these data was the same whether or not one accounted for dilution of exogenous substrate with endogenous cholesterol esters. For future characterization of CEH activity, that fraction with the greatest specific and total activity ($14{,}000\,g_{max}$ pellet) was used. In addition, because many publications concerned with hormonal regulation of adrenal CEH have used the soluble cytosol [9, 10, 14, 35, 36], CEH activity in this subcellular fraction was also evaluated.

Effects of pH. As indicated in Fig. 1, CEH activity was optimum at pH 7.0 for both the F4 and F6, although a somewhat broader pH optimum was noted for F6 CEH activity. In these studies, there was no measured F4 CEH activity below pH 7.0. However, later tests measured low activity at pH 6.0 (see Experiment II below). For subsequent tests, pH 7.0 was used for both subcellular fractions.

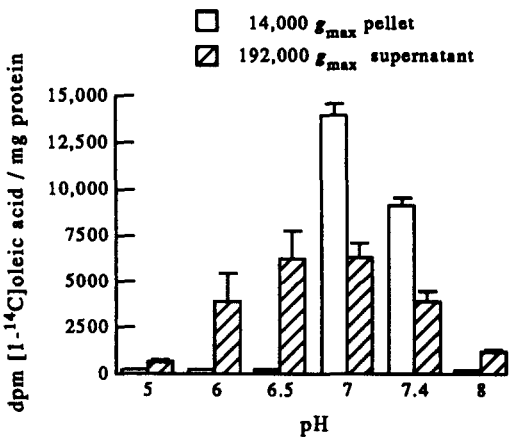


Fig. 1. Effects of pH on CEH activity in the $14{,}000\,g_{max}$ pellet (F4; 195 μg protein), and in the $192{,}000\,g_{max}$ supernatant (F6, 343 μg protein) of porcine adrenal tissue preparations. Assays were conducted for 20 min (F4) or 10 min (F6) at 37°C and with addition of 200,000 dpm labeled and 10 μg unlabeled (33 μM) cholesteryl oleate. Data represents the mean ± SEM of two (F4) or three (F6) different tissue preparations with triplicate determinations conducted for each preparation, subcellular fraction, and at each pH.

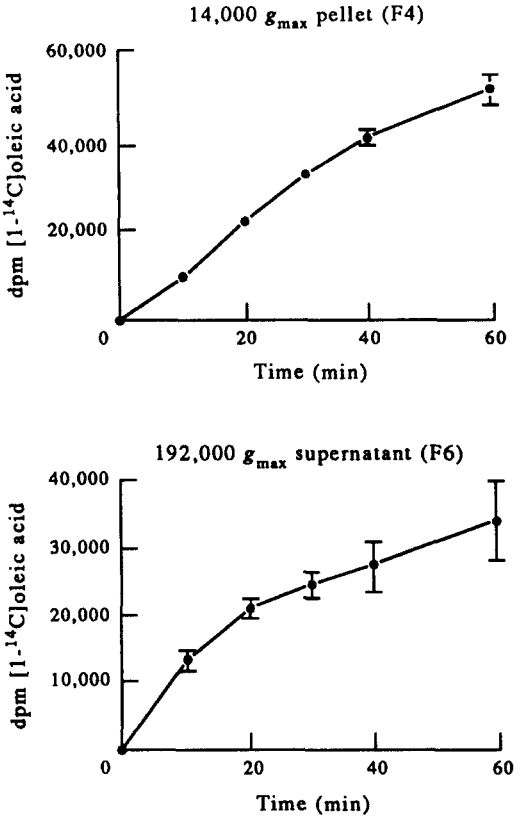


Fig. 2. Effects of time on product formation for porcine adrenal CEH activity in the 14,000 g_{max} pellet (F4; 88.3 μg protein) and in the 192,000 g_{max} supernatant (F6, 127 μg protein). Assays conducted at pH 7.0 37°C, and after addition of 200,000 dpm labeled and 10 μg unlabeled (33 μM) cholesteryl oleate. Data represent the mean ± SEM of two different tissue preparations with triplicate determinations conducted for each preparation, subcellular fraction, and time.

Effects of time on product formation. Product formation (1012 dpm/mg protein/min) by F4 was almost constant for a 40-min period (Fig. 2) and declined thereafter (530 dpm/mg protein/min). For F6, product formation occurred at its greatest rate from 0–10 min (1331 dpm/mg protein/min). This rate declined by 42% between 10 and 20 min. For most subsequent tests and experimental samples, a 20-min incubation period was used for F4 and a 10-min incubation for F6.

Linearity with protein. For F4, product formation was linear up to 110 μg protein at a lower substrate concentration (4.2 μM), and up to 220 μg at a higher substrate concentration (247 μM). Beyond 220 μg protein an inhibitory effect was apparent (Fig. 3). For F6 product formation was linear for up to 500 μg protein at both substrate concentrations (Fig. 3).

Equilibration of exogenous labeled substrate delivered in acetone with endogenous cholesterol esters. It was apparent from the subcellular fractionation study that removal of the floating lipid layer at each centrifugation did not remove all endogenous cholesterol esters (data not shown). Hence, this study was conducted to determine what influence high and potentially variable endogenous levels of cholesterol esters would have on measurement of conversion of exogenous cholesteryl oleate ([1-^{14}C]oleate) to [1-^{14}C]oleic acid. The rationale of the study was that if both exogenous and endogenous cholesterol esters can compete with labeled substrate for the enzyme, then as unlabeled cholesterol esters increased, fewer dpm of labeled product would be formed. Data in Fig. 4 dramatically illustrate that exogenous cholesteryl oleate added in acetone effectively competed with labeled substrate. In both F4 and F6 fractions, conversion of labeled substrate to product diminished to 5–12% of that occurring without unlabeled cholesteryl oleate. On the contrary, endogenous cholesterol esters added in heated tissue preparations (Fig. 4), were associated with only a 13% decrease in conversion of labeled substrate. Such data suggest that cholesteryl oleate dissolved and delivered in acetone, serves as a preferential substrate, and endogenous cholesterol esters do not compete to a significant extent with those delivered in acetone. Therefore, when measuring CEH activity using this procedure, one does not need to consider the dilution and competition effects of potentially high and variable endogenous substrate.

Saturating substrate concentrations: determination of K_m and V_{max}. Velocity for both F4 and F6 increased rapidly and linearly between approx. 1 and 20 μM (Figs 5 and 6). Thereafter, the rate of product formation slowed as the enzymes approached saturation. Lineweaver–Burke plots (Figs 5 and 6) to measure K_m and V_{max} demonstrated a significant ($P < 0.01$) linear regression but appeared suspiciously curvilinear or biphasic. Replot of these data using Scatchard analyses (Figs 7 and 8) clearly indicated the presence of either multiple enzymes or multiple catalytic sites catalyzing the same reaction [37]. The program LIGAND indicated for both F4 and F6, the presence of lower K_m lower velocity enzymes (catalytic sites), and higher K_m higher velocity enzymes (catalytic sites). Such data suggested that saturation curves and subsequent Scatchard analyses be conducted on all experimental samples. However, the difficulty of this assay and time

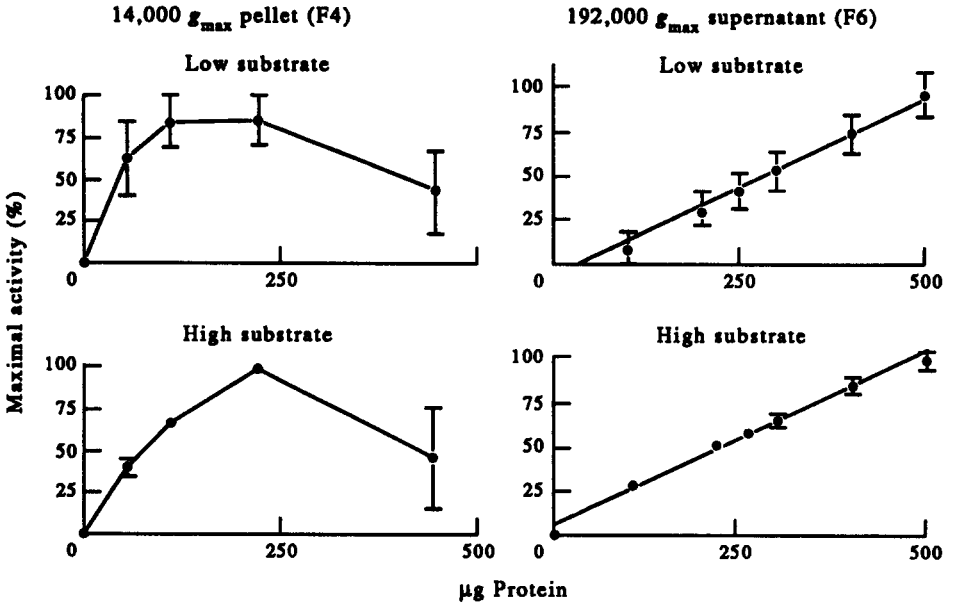


Fig. 3. Effects of increasing quantities of porcine adrenal subcellular fraction protein on rate of hydrolysis of cholesteryl oleate ([1-^{14}C]oleate) to [1-^{14}C]oleic acid. For the 14,000 g_{max} pellet (F4), incubations were conducted at pH 7.0, 37°C for 20 min, and in presence of either 4.2 or 247 μM substrate. For the 192,000 g_{max} supernatant (F6), incubations were conducted at pH 7.0, 37°C, for 10 min, and in presence of either 0.3 or 261 μM substrate. Data represent the mean ± SEM of two different tissue preparations with triplicate determinations conducted for each preparation, subcellular fraction, and quantity of protein.

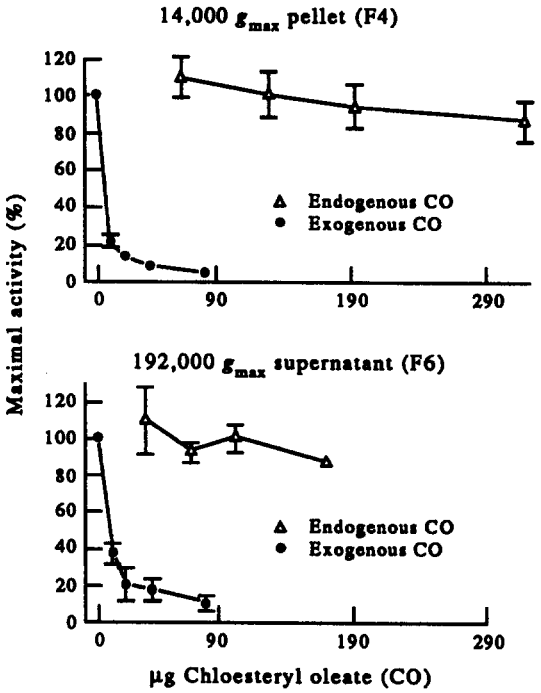
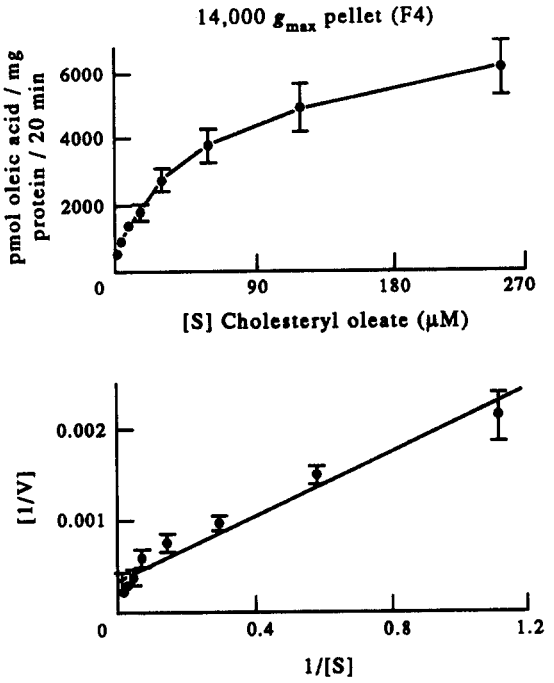


Fig. 4. Effects of unlabeled cholesteryl oleate delivered in acetone (exogenous) or cholesterol esters added as a heat-treated volume of additional subcellular fraction (endogenous) on the conversion of cholesteryl oleate ([1-^{14}C]oleate) to [1-^{14}C]oleic acid. Assays were conducted with 14,000 g_{max} pellet (F4, 268 μg protein) and 192,000 g_{max} supernatant (F6, 193 μg protein), at pH 7.0, 37°C, and for 20 min after addition of 207,000 dpm labeled cholesteryl oleate (3.2 μM). Each data point represents the mean ± SEM of two different tissue preparations with triplicate determinations for each preparation, subcellular fraction, and treatment combination.

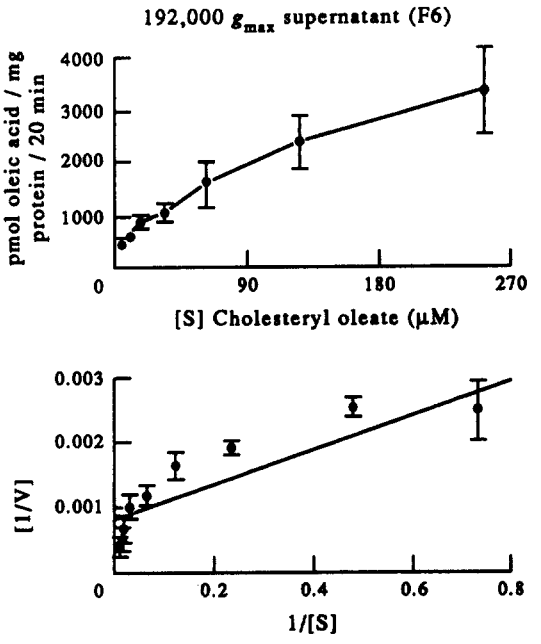
required for such analyses made such an approach impractical. As a compromise, each experimental sample was measured at a very low and a very high substrate concentration (0.4 and 241 μM) in an attempt to measure treatment effects for these two enzymes (catalytic sites) in each subcellular fraction. Using K_m and V_{max} values presented in Figs 7 and 8 and the Michaelis–Menten equation, it was computed that for F4 at the low substrate concentration (0.4 μM), the contribution to product formation by enzyme 1 is 91%. At the high substrate concentration, 82.8% of product formation results from activity of enzyme 2. For F6 at a substrate concentration of 0.4 μM, 96.6% of product formation results from activity of enzyme 1, whereas at a substrate concentration of 241 μM, 86.1% of product formation originates from enzyme 2.

Experiment I. Adrenal weights, which have been reported previously for this study [22], were unaffected by any of the treatments, and had an average weight across all treatments and time periods of 3.55 ± 0.07 g. Plasma ACTH, cortisol, and PRL have also been previously reported [22]. ACTH was elevated 5-fold after the 30-min restraint whereas plasma cortisol increased 1.7-fold ($P < 0.05$). Both had returned to, or below, prestressor concentrations by 2 h after stressor initiation. ACTH injection produced supraphysiological concentrations of



[S] = Cholesteryl oleate (μM)
V = pmol Oleic acid / mg protein / 20 min

Fig. 5. Effects of increasing substrate concentrations on porcine adrenal CEH activity in the 14,000 g_{max} pellet (F4). Top frame represents saturation curve, and bottom frame the Lineweaver–Burke transformation of original data. Cholesteryl oleate substrate increased from 0.89–260 μM with a nearly constant ratio of labeled (3021–840,000 dpm) to unlabeled (0.29–82 μg) cholesteryl oleate. Incubations were conducted at pH 7.0, 37°C, 20 min, and at protein concentrations of 65–260 μg. Each data point represents the mean ± SEM of four separate tissue preparations with triplicate determinations conducted for each preparation and substrate concentration.



[S] = Cholesteryl oleate (μM)
V = pmol Oleic acid / mg protein / 20 min

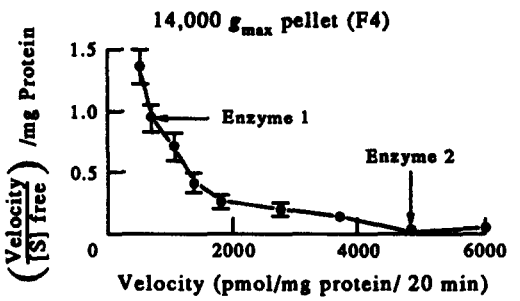
Fig. 6. Effects of increasing substrate concentrations on porcine adrenal CEH activity in the 192,000 g_{max} supernatant (F6). Top frame represents saturation curve, and bottom frame the Lineweaver–Burke transformation of original data. Incubations conducted as indicated in Fig. 5 legend and using 220–380 μg F6 protein. Each datum point represents the mean ± SEM of three separate tissue preparations with triplicate determinations conducted for each preparation and substrate concentration.

ACTH at 0.5 h (20.39 ± 4.86 ng/ml), 2 h (6.41 ± 2.11 ng/ml), and 5 h (5.24 ± 2.28 ng/ml). Plasma cortisol was elevated 1.7-fold at only 0.5 h. Plasma PRL concentrations were reduced 53% ($P < 0.05$) by CB154 treatment.

ANOVA of CEH data (pmol/min/paired adrenals) for F4 at the low substrate concentration showed significant treatment effects ($P = 0.003$) but, neither time ($P = 0.56$) nor interaction effects ($P = 0.09$). Because of the lack of time-of-measurement and interaction effects, data were combined for each treatment across all time periods (Fig. 9). At a low substrate concentration, neither ACTH nor CB154 by themselves produced significant treatment effects on F4. However, in combination, a dramatic 63% decrease in F4 CEH activity was readily apparent. This decrease occurred at all three time periods (data not shown). For F6 at the low substrate concentration, there were no treatment ($P = 0.22$), time ($P = 0.27$), or interaction ($P = 0.93$) effects. However, the 49% decrease in CEH activity after concomitant administration of ACTH and CB154 was significant ($P = 0.03$) by *a priori* analysis.

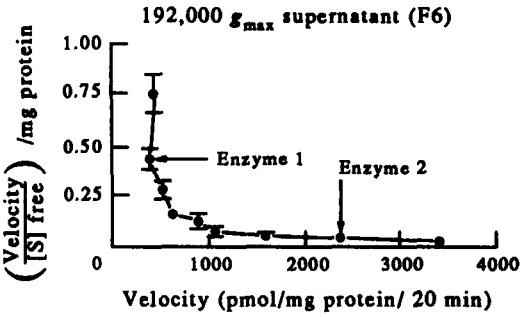
ANOVA of CEH data for F4 and F6 at the high substrate concentration revealed neither treatment, time, nor interaction effects ($P > 0.05$) (Fig. 9). Neither were *a priori* tests significant. Cholesterol ester concentrations were remarkably constant ($P > 0.05$) among treatments and time periods and had an overall mean of 22.59 ± 0.49 μg/mg tissue.

Experiment II. Experiment I CEH activity was that measured at neutrality (pH 7.0). To investigate the possibility that acidic (pH 6.0) CEH activity might be regulated by stressor associated hormonal perturbations a second study was conducted. In this study, basal plasma immunoreactive ACTH and cortisol did not differ before stressor application (Table 1). However, after the 30-min restraint and just



	K_m (M)	V_{max} (pmol / mg protein / 20 min)
Enzyme 1	$9.398 \pm 1.12 \times 10^{-7}$	1017.7 ± 88.4
Enzyme 2	$8.857 \pm 1.521 \times 10^{-5}$	6697.3 ± 928.2

Fig. 7. Scatchard plot of saturation data for 14,000 g_{max} pellet illustrated previously in Fig. 5. Results for V_{max} and K_m represented in tabular form were obtained via use of computer program LIGAND.



	K_m (M)	V_{max} (pmol / mg protein / 20 min)
Enzyme 1	$3.377 \pm 1.097 \times 10^{-7}$	456.3 ± 11.4
Enzyme 2	$2.776 \pm 1.403 \times 10^{-4}$	6075.8 ± 3508

Fig. 8. Scatchard plot of saturation data for 192,000 g_{max} supernatant illustrated previously in Fig. 6. Results for V_{max} and K_m represented in tabular form were obtained via use of computer program LIGAND.

minutes before killing, plasma ACTH was elevated 9-fold above control concentrations ($P < 0.05$), and plasma cortisol increased 4-fold ($P < 0.05$). Plasma PRL also increased (2.5-fold). Hence, activation of the pituitary–adrenal axis had occurred. Acidic adrenocortical CEH activity measured in pigs killed after the 30-min stressor did not differ between treatment groups

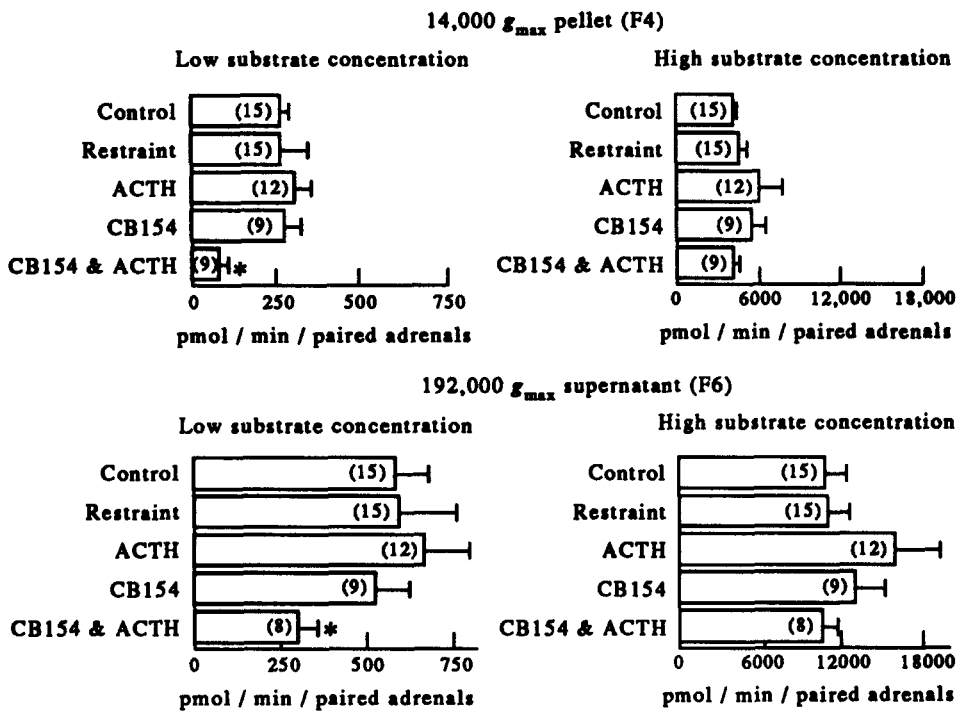


Fig. 9. Effects of ACTH, restraint, and bromocriptine (CB154) on neutral (pH 7.0) CEH activity in two subcellular fractions of the porcine adrenal. CEH activity was measured at low (0.4 μM) and high (241 μM) substrate concentrations. Barrows were killed for CEH measurement at 0.5, 2, and 5 h after ACTH injection (0.4 μg/kg body weight) or beginning of 30-min restraint. Since there were no time effects or treatment × time interaction, data within each treatment were combined across all time periods. The number of barrows per treatment group are in parentheses. Each bar represents the mean ± SEM. Bars with asterisk (*) are significantly different ($P < 0.05$) from control animals.

Table 1. Effects of 30-min restraint on plasma hormonal concentrations in barrows

Treatment	Time	(n)	ACTH (pg/ml)	Cortisol (ng/ml)	PRL (ng/ml)
Control	−10 min	(6)	63.6 ± 20.1	30.6 ± 6.5	1.4 ± 0.2
	+30 min	(6)	36.1 ± 10.4	14.5 ± 4.0	1.1 ± 0.1
Restrained	−10 min	(6)	40.8 ± 4.5	26.5 ± 3.1	1.5 ± 0.2
	+30 min	(6)	363.6 ± 69.9*	74.7 ± 5.1*	5.2 ± 0.9*

*Hormonal concentrations significantly greater ($P < 0.01$) than those at −10 min in same treatment and from control animals at both time periods. Blood samples were obtained 10 min prior to or 30 min after initiation of restraint or at similar time intervals in control barrows. Data represent mean ± SEM.

Table 2. Effects of 30-min restraint and associated hormonal perturbations on acidic CEH activity

Subcellular fraction	Treatment	Substrate concentration: Low (0.4 μM)	Substrate concentration: High (241 μM)
		CEH activity (pmol/min/paired adrenals)	
14,000 g_{max} pellet (F4)	Control (5)	143.3 ± 54.7	1968.1 ± 568.9
	Restraint (6)	95.7 ± 49.5	1356.7 ± 447.9
192,000 g_{max} supernatant (F6)	Control (5)	499.3 ± 81.3	3031.7 ± 844.4
	Restraint (6)	464.6 ± 85.5	2432.0 ± 588.8

CEH activity measured at pH 6.0. There were no significant differences ($P > 0.05$) between treatments for CEH activity in either subcellular fraction and at either substrate concentration. Data represent mean ± SEM of number of pigs shown in parentheses.

for either F4 or F6 CEH activity when measured at either substrate concentration (Table 2).

Experiment III. This experiment was conducted to determine if a longer restraint period (45 min) or repeated daily exposures to this stressor would alter neutral CEH activity. This stressor duration was used since longer periods (1.5 or 3 h) were not well tolerated by some of the pigs, and hence for animal welfare considerations were not used. A single restraint produced maximal ACTH concentrations (the greatest concentration attained during the sampling period) 4.5-fold greater than controls and 1.6-fold greater ($P < 0.05$) than the last of nine daily restraints (Table 3). However, the last of nine restraints still elicited an ACTH response above that of control pigs ($P < 0.05$). Twenty-four hours after the last exposure, pigs receiving eight daily restraints had maximal plasma ACTH concentrations, which did not differ from those of control pigs. Plasma cortisol concentrations (Table 3) during one restraint reached concentrations 52% greater than those of control pigs ($P < 0.05$). After nine repeated daily restraints, however, maximal cortisol concentrations were not above those of control pigs nor below those of pigs subjected to a single restraint ($P > 0.05$). Commensurate with elevated plasma ACTH and cortisol during the first restraint exposure, there was a 148% increase ($P = 0.003$) in F4 CEH activity measured at a high substrate concentration and, concomitantly, an 80% decrease ($P = 0.003$) in F6 CEH activity measured at a low substrate concentration (Fig. 10). Adrenal cholesterol ester concentrations were decreased 23% ($P = 0.024$) 1 h after termination of this initial 45-min restraint (Table 3). Subsequent to 9 daily 45-min restraint

Table 3. Effects of a single or of repeated daily exposure to a 45-min restraint on plasma hormonal concentrations and adrenal cholesterol esters in castrated male pigs

Treatment*	n	Maximal** plasma ACTH (pg/ml)	Maximal** plasma cortisol (ng/ml)	μg Cholesterol esters (mg tissue)
Control	5	88.0 ± 22.7^{c}	67.9 ± 11.1^{a}	12.4 ± 0.6^{a}
One restraint	4	487.5 ± 120.3^{a}	103.4 ± 13.2^{b}	9.6 ± 0.8^{b}
Eight restraints	5	53.8 ± 11.1^{c}	59.1 ± 4.9^{a}	12.6 ± 1.0^{a}
Nine restraints	5	184.8 ± 38.7^{b}	81.0 ± 6.1^{a,b}	12.5 ± 1.1^{a}

*All measures taken on day 9 of the study. For animals receiving 8 daily restraints, this is 24 h after the end of the last restraining period. Adrenal glands were obtained 1 h after termination of the 45-min stressor.

**Data represent the maximal concentration of hormone attained during the sampling period. Blood samples were taken at −20, +3, +10, +20, +40, and +45 min with reference to initiation of restraint and at similar intervals in control animals.

Data represent the mean ± SEM. Means with different letter superscripts for a given hormone are significantly different ($P < 0.05$).

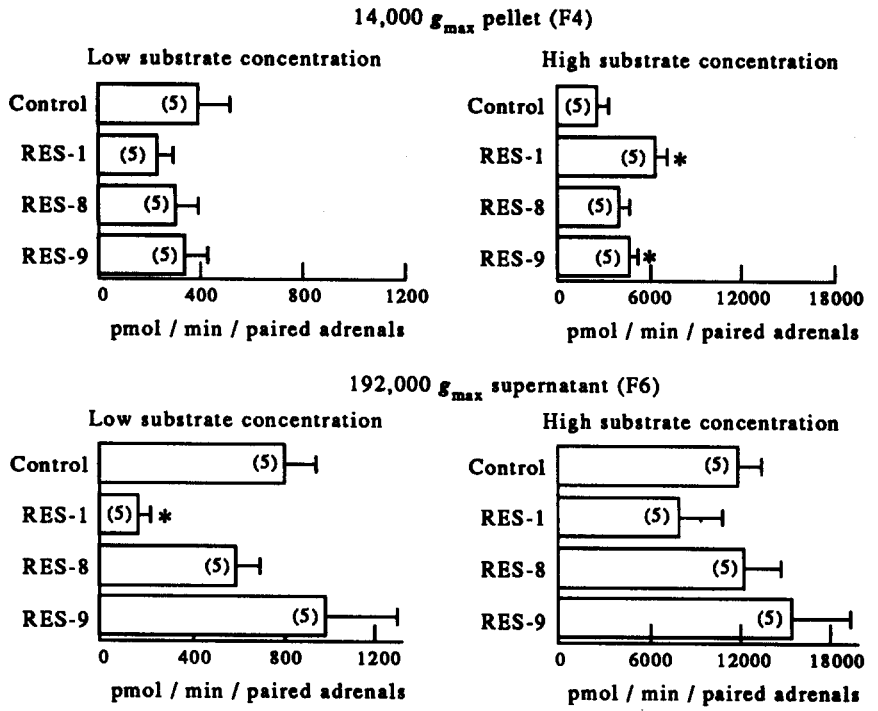


Fig. 10. Effects of 45-min restraint stressor and associated hormonal changes on porcine neutral CEH activity in two subcellular fractions. Castrated male pigs received a single treatment period and were killed 1 h later (RES-1), or 8 daily treatments and were killed 24 h later (RES-8), or 9 daily treatments and were killed 1 h later (RES-9). CEH activity was measured in each subcellular fraction at low (0.4 μM) and high (241 μM) substrate concentrations. Each bar represents the mean ± SEM. The number of barrows per treatment group is in parentheses. Means with an * are significantly different ($P < 0.05$) from that of control pigs.

periods, F4 CEH activity measured at the high substrate concentration was still increased 75% above that of control pigs ($P = 0.03$).

DISCUSSION

Data presented herein represent the first characterization of CEH for the swine adrenal, and perhaps more importantly, the first account of *in vivo* hormonal regulation of this adrenal enzyme in a species evolutionarily more advanced than rodents. Tests conducted to measure subcellular distribution and characteristics of porcine adrenal CEH revealed properties that resembled those that exist in rats and guinea pigs [9, 11, 12, 26, 28, 36]. Initial studies suggested that the greatest specific and total activity for porcine adrenal CEH were present in the 14,000 g_{max} pellet (F4), and only a modest amount of activity was present in the 192,000 g_{max} supernatant or soluble cytosol (F6). However, subsequent results in the current studies indicated that, under more optimal conditions of time and protein concentrations for each subcellular fraction, CEH specific activity was greater in the cytosol than in the 14,000 g_{max} pellet (Fig. 9). Such information suggests that CEH activity measurements need to be optimized for each subcellular fraction before accurate subcellular fractionation studies can be conducted.

Problems with endogenous substrate effects on an exogenous labeled substrate and competition for the enzyme measured need to be considered when conducting enzymatic analyses with impure preparations [37]. Problems with substrate concentration-associated changes in enzymatic activity can be avoided by using saturating substrate concentrations which permit maximal velocity (V_{max}) [37]. Problems with variable substrate dilution can be alleviated by either removing endogenous substrate or by measuring its levels so that the specific activity of substrate can be validly calculated. Since we were unable to remove endogenous cholesterol esters, the above-noted studies dealing with equilibration of exogenous cholesteryl oleate delivered in acetone with endogenous cholesterol esters were conducted. The data strongly suggest that this method of substrate addition obviates problems with dilution of exogenous cholesterol esters by endogenous cholesterol es-

ters. This apparent absence of mixing of cholesterol esters delivered in acetone has been suggested before [6], but the current data are the first direct evidence for such a phenomenon in adrenal homogenates. These data do not mean that endogenous cholsterol esters are a poor substrate for CEH activity, only that under the conditions of our *in vitro* system, cholesterol esters delivered in acetone are preferentially used. Because of these observations, only the specific activity of the exogenous cholesterol esters was used in calculating mass of product formed.

Experiments conducted to measure appropriate concentrations of substrate needed to saturate porcine adrenal CEH and achieve maximal velocity produced surprising results. The Lineweaver–Burke plots appeared markedly curvilinear or biphasic, and Scatchard analyses clearly demonstrated curvilinearity. Such plots can be interpreted to represent multiple enzymes with different K_m and V_{max} that catalyze the same reaction [37]. However, as with curved Scatchard plots for receptors [38], such data also could represent multisite enzymes that have differing affinities for the substrate, or which display negative cooperativity. The data cannot distinguish among these alternatives [37]. For discussion purposes, it is assumed that two enzymes are represented. Two CEH activities existed in F4 and in F6. V_{max} for the respective CEH activities in the two subcellular fractions did not differ significantly, but K_m for the high velocity enzymes was different between F4 and F6. Similar activities in the two subcellular fractions were regulated differently. Curvilinear Eadie–Hofstee plots from CEH activity in rat adrenal have been described [39], and curvilinear Lineweaver–Burke plots for CEH activity in the outer zone of the guinea pig adrenal were present but not discussed [11].

It would have been best to measure all experimental samples at multiple substrate concentrations to accurately measure changes in velocities and K_m for both enzymatic activities in both subcellular fractions. The procedures used were a pragmatic compromise to obtain estimates of velocities for these enzymatic activities. The preferential use of cholesteryl oleate delivered in acetone, and the previously noted calculations, suggests that such procedures are valid. All previously published papers concerned with regulation of rodent adrenal CEH activity have used a single substrate concentration.

The potential exists that one or more of these enzymatic activities may represent triacylglycerol lipase activity. Besides its ability to stimulate a rapid decrease in cholesterol esters in rodents via increased CEH activity, ACTH also stimulates a parallel decrease in triacylglycerols [40] that occurs coincident with an activation of triacylglycerol lipase [39, 41]. The bovine adrenal cortex also possesses triacylglycerol lipase activity [42]. Lipase activity in rat adrenal uses trioleylglycerol and cholesteryl oleate equally well as substrate and may account, in part, for the above-noted curvilinear Eadie–Hofstee plots [39]. The presence of two molecular forms of CEH activity in rat and bovine adrenal glands [39, 43] and two triacylglycerol lipase activities in bovine adrenals [42] agree with the possibility that the current results represent two or more enzymes with cholesterol ester hydrolyzing activity. It is also possible that the different CEH activities originate from different types of adrenocortical cells since the entire adrenal cortex was used. However, morphometric data indicated that cells of the porcine zona fasciculata constituted approx. 82% of the adrenal cortex, with glomerulosa and reticularis cells representing 15 and 3%, respectively [44]. Hence, in the current studies, it is probable that most CEH activity is from fasciculata cells.

Regulation of these CEH activities by hormones or stressor-associated perturbations appeared complex: (1) ACTH administration by itself had no effect on neutral CEH activity, (2) 30-min restraint stressor and associated hormonal changes had no effect on neutral or acidic CEH activities, (3) more prolonged restraint (45 min) altered CEH activity differentially in the F4 (increased activity) and F6 (decreased activity) subcellular fractions, (4) ACTH administration to bromocriptine-induced hypoprolactinemic pigs produced dramatic reductions in CEH activities measured at low substrate concentrations in both F4 and F6. Absence of ACTH and 30-min stressor effects on CEH are contrary to much of the data in rodents [9, 10, 28, 36, 39, 45, 46]. They also differ from *in vitro* results with bovine adrenals that demonstrated CEH activity is stimulated by a cAMP-dependent protein kinase with the inference that this occurs *in vivo* after ACTH activation of adenylate cyclase [35, 47]. Current data may indicate that CEH activities are operating at maximal rates under basal conditions as has been suggested for guinea pig adrenals [12].

These data could indicate that a certain threshold of duration and/or magnitude of stimulus must be surpassed before CEH activity is altered. Once this occurs, cellular mechanisms occur—perhaps involving protein synthesis—that differentially increase or decrease CEH activity depending on the cellular location of the enzyme. The data also indicate that hormones other than, or in addition to, ACTH are involved in the regulation of porcine adrenal CEH activity. Similar results have been reported for rodents. For example, administration of ACTH to rodents does not always lead to activation of adrenal CEH in situations of increased plasma corticosterone [14, 16]. A dose–response of CEH activity to ACTH indicated high concentrations of ACTH were needed for significant activation of adrenal CEH [16]. There is also evidence in rodents for a pituitary factor other than ACTH—which is also derived from the proopiomelanocortin precursor—that is involved in regulation of adrenal CEH activity [14, 15].

The current data also suggest a regulation for porcine CEH for which there is no precedence in rodents. Firstly, there is no previous evidence for stressor effects on neutral CEH activity in the $7500\,g_{min}$–$14{,}000\,g_{max}$ pellet that usually contains mitochondria and lysosomes [28, 48]. Studies concerned with ACTH or stressor-associated increases in CEH have dealt almost exclusively with cytosolic CEH [9, 10, 12, 14–16, 26, 28, 36, 39, 45, 46]. In one study, stress increased neutral microsomal CEH activity in guinea pigs [11]. Although considerable neutral CEH activity has been measured in a rodent particulate fraction similar to the $14{,}000\,g_{max}$ pellet of the current studies [9, 28] hormonal regulation of this neutral CEH activity has not been explored.

Secondly, there is no previous evidence for stressor-associated decreases in CEH activity such as occurred in the present studies (Experiment III). It is highly unlikely that this reduction in CEH activity can be explained by dilution of exogenous labeled substrate with endogenous cholesterol esters. Not only did our studies indicate a preferential use of exogenous substrate delivered in acetone, but also for this treatment group there was a significant decrease in endogenous cholesterol esters. The physiological significance of this decreased CEH activity at a time when the adrenal is synthesizing and secreting large amounts of cortisol is unknown and must remain speculative.

Thirdly, the data suggest an involvement of PRL or of dopamine in the regulation of porcine adrenal CEH activity. Bromocriptine—and associated hypoprolactinemia—by itself lacked effect on adrenal CEH activity. However, this treatment allowed ACTH to induce dramatic reductions in CEH activities associated with low K_m and V_{max}. Such results may reflect a requirement for PRL to protect discrete CEH activities from inhibitory effects of high concentrations of ACTH. PRL receptors are present in the porcine adrenal [23]; in other species, PRL stimulates adrenal function [19–21]. Alternatively, a potential direct effect of bromocriptine on adrenocortical CEH must be considered. It is possible that the concentrations of dopamine agonist attained were sufficient to reveal inhibitory effects of high ACTH concentrations on discrete CEH activities. Dopamine D2 receptors are present in adrenocortical tissue of other species [49, 50], and dopamine inhibits aldosterone production in several species [50–53]. At high concentrations (6.5×10^{-4} M), dopamine inhibited *in vitro* corticosterone production, but at lower concentrations, increased ACTH-stimulated corticosterone production [54]. Effects of dopamine may also be indirect via a variety of potential effects on the autonomic nervous system [55]. Plasma catecholamines were not measured in the current studies; but in swine, both epinephrine and norepinephrine directly stimulate cortisol and aldosterone secretion from the adrenal [56]. Hence, observed effects of bromocriptine and associated hypoprolactinemia on CEH activities may be explained by a variety of mechanisms. Knowledge of that mechanism that actually pertains must await further investigation.

In summary, the porcine adrenal has multiple cholesterol ester hydrolyzing activities in at least two subcellular fractions, the soluble cytosol and the $14{,}000\,g_{max}$ pellet. Regulation of these different CEH activities appears complex and undoubtedly involves factors other than ACTH alone. A duration-dependent ability of restraint stressor to affect CEH activity suggests that a certain threshold must be exceeded before effects are apparent. Clearly, however, correct stimulus strength increased CEH activity located in the $14{,}000\,g_{max}$ pellet, which in turn was associated with reduced cholesterol esters and increased plasma cortisol. A concomitant reduction in CEH activity in another subcellular fraction, or during ACTH stimulation of hypoprolactinemic pigs, suggests a complex

regulation of CEH activities that may depend upon their subcellular location, substrate specificity, or the specific cell population within the adrenal cortex in which a particular CEH activity exists.

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