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Expression of Steroidogenic Enzyme Messenger Ribonucleic Acid and Cortisol Production in Adrenocortical Cells Isolated From Halothane-Sensitive and Halothane-Resistant Pigs

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Abstract Stress susceptibility in pigs is inherited by a single recessive gene (*Hal*^{n}), and homozygous individuals can be identified by exposure to halothane anesthesia. Previous studies have shown that in stress-susceptible pigs, exposure to a high ambient temperature resulted in a twofold increase in corticotropin (ACTH) and lower plasma cortisol. To determine whether there is a fundamental difference in adrenocortical function between halothane-sensitive (HAL-S) and halothane-resistant (HAL-R) pigs, independent of other factors influencing the hypothalamic–pituitary–adrenal (HPA) axis, we compared cortisol responses to ACTH and 8-bromo-cyclic AMP (8-Br-cAMP) in HAL-S and HAL-R pig adrenocortical cells in vitro. We also determined directly the accumulation of four different mRNAs encoding cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$), 17α-hydroxylase cytochrome P450 ($P450_{17\alpha}$), 21-hydroxylase cytochrome P450 ($P450_{c21}$) and 11β-hydroxylase cytochrome P450 ($P450_{11\beta}$) in HAL-S pig adrenal cells and compared them to HAL-R pigs. A time- and dose-dependent increase in medium content of cortisol and cAMP was observed after ACTH treatment. 8-Br-cAMP also caused a time- and dose-dependent increase in cortisol production in the medium. Addition of ACTH or 8-Br-cAMP to HAL-S and HAL-R male Lanyu small-ear miniature pig adrenocortical cells increased cortisol production in a dose- and time-related manner. However, cells isolated from HAL-S pigs had a lower cortisol production in response to ACTH or 8-Br-cAMP compared to those from HAL-R pigs. Treatment of cultured cells with 8-Br-cAMP (0.5 mM) for 18 h resulted in a significant increase in $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNA levels. In the absence of 8-Br-cAMP, the four genes were expressed constitutively in both HAL-S and HAL-R pig adrenal cells. Densitometric scanning of the autoradiograph indicated that the relative amounts of $P450_{scc}$ and $P450_{17_\alpha}$ mRNAs in HAL-S pig adrenal cells were between 48% and 53% of those detected in HAL-R pig adrenal cells ($P < 0.05$). No difference in the amounts of $P450_{c21}$ and $P450_{11\beta}$ was seen in HAL-S and HAL-R pig adrenal cells. Addition of 8-Br-cAMP (0.5 mM) resulted in a uniform increase in the levels of all four P450 mRNAs in both HAL-S and HAL-R pig adrenal cells. However, the amounts of $P450_{scc}$ mRNA in HAL-S pig adrenal cells were 67% ($P < 0.05$) of those measured in HAL-R pig adrenal cells, whereas the amounts of $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNAs were similar in these cells. Our data suggest an HPA axis defect in HAL-S pigs at the adrenal level. This defect appears to be at the level of P450scc gene expression, which could be partially related to reduced cortisol production by ACTH stimulation. J. Cell. Biochem. 79:58–70, 2000.

Key words: pig adrenocortical cells; cortisol; P450 gene expression; halothane

Stress susceptibility in pigs is inherited by a single recessive gene (*Hal*^{n}), and homozygous individuals can be identified by exposure to halothane anesthesia. The HAL-locus with two alleles *N* and *n* [Smith and Bampton, 1977] resides on pig chromosome 6. Pigs of genotypes *NN* and *Nn* are not sensitive to halothane, whereas pigs of genotype *nn* are sensitive to halothane. Under stress conditions, such as transport, high ambient temperature, exercise, fighting, service, and parturition, the halothane-sensitive *nn* pigs develop porcine stress syndrome, which is characterized by malignant hyperthermia (MH) and muscle rigidity [Nel-

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son, 1973]. The acute reaction to stress may be followed by sudden death within minutes. The stress-induced death of such pigs is but one aspect of economic loss caused by the syndrome. An equally serious problem is that the same reaction can be triggered when a pig experiences acute stress before slaughter, resulting in pale, soft, exudative pork in large segments of the carcasses of susceptible animals [Nelson, 1973]. Subsequent studies indicated that the stress-induced MH in halothane-sensitive pigs is caused by a single mutation ($Arg^{615} \rightarrow Cys^{615}$) in the ryanodine receptor-Ca^{2+} release channel in the skeletal muscle sarcoplasmic reticulum [Fujii et al., 1991]. The identity of the pig MH (*Hal*) gene and the ryanodine receptor gene has also been demonstrated [Mickelson et al., 1989; Otsu et al., 1991]. However, it is unknown at present how this mutation in the ryanodine receptor results in MH susceptibility.

A vital function of the adrenal cortex is its responsiveness to stress. It is well established that, through the secretion of corticotropin-releasing factor and corticotropin (ACTH), an acute stress can stimulate steroidogenesis in cortical cells, leading to an elevation of plasma glucocorticoids which, in turn, causes many metabolic and physiological changes to adapt the organism to the changing environment. Changes in serum cortisol are considered a valid indicator of stress in pigs [Becker et al., 1985]. Abnormal glucocorticoid secretion in response to stressors can result from alterations in any of the modulating factors operating at the levels of the hypothalamus–pituitary–adrenal (HPA) axis. An altered pituitary-adrenal function has been noted in pigs of certain genetic strains that often die when exposed to stressful situations [Marple et al., 1972]. Such stress-susceptible pigs were shown to have higher levels of circulating ACTH at rest without a corresponding increase in plasma adrenal corticoids [Marple et al., 1972]. Subsequent work by Marple and Cassens [1973] demonstrated that the metabolic clearance rate (MCR) and turnover rate of cortisol was elevated in stress-susceptible pigs. However, in pigs of the same age, sex, and genetic origin, the MCR of cortisol was similar for high and low ACTH responders [Hennessy et al., 1986]. Studies by Zhang et al. [1990] further indicated that the differences in adrenocortical response to ACTH are primarily of adrenal origin. These studies were all in vivo studies and, thus, the presence of extraadrenal factors complicates the interpretation of the results.

Cortisol secretion from the adrenal cortex is generally regarded as being under the control of ACTH. ACTH acutely induces the mobilization of cholesterol from its storage sites to the inner mitochondrial membrane and, thus, the removal of the six-carbon side chain from cholesterol to form pregnenolone, a reaction catalyzed by cholesterol side-chain cleavage cytochrome P450 (P450scc), in a matter of minutes or even seconds [Jefcoate et al., 1987; Simpson and Waterman, 1988]. After conversion of pregnenolone to progesterone by the 3β-hydroxysteroid dehydrogenase–isomerase enzyme complex, hydroxylation by 17α-hydroxylase cytochrome P450 ($P450_{17\alpha}$) and then by 21-hydroxylase cytochrome P450 ($P450_{c21}$) yields 11-deoxycortisol. 11-Deoxycortisol is then further hydroxylated at the 11β position by 11β-hydroxylase cytochrome P450 ($P450_{11\beta}$) to form cortisol. The chronic response to ACTH in the adrenal cortex involves increased transcription of the genes encoding these hydroxylases [Waterman et al., 1986; Simpson and Waterman, 1988; Imai et al., 1990]. The effects of ACTH are thought to be mediated by cAMP [Saez et al., 1981; Kramer et al., 1984; Zuber et al., 1986], presumably involving cAMP-dependent protein kinase [Handler et al., 1988].

Because little is known about the cause of stress susceptibility in pigs, we believe that the mechanism of decreased cortical response to ACTH needs to be considered. In particular, no studies have directly addressed the function of adrenocortical cells from stress-susceptible pigs. In an effort to gain further understanding of the cellular and molecular basis of adrenocortical function of pigs sensitive to halothane anesthesia that confers to stress-susceptibility, we have used an in vitro system. We first examined the time–dose dependency of the effect of ACTH on the secretion of cortisol and cAMP and on the accumulation of the four different mRNAs encoding $P450_{scc}$, $P450_{c21}$, $P450_{17\alpha}$ and $P450_{11\beta}$in isolated pig adrenocortical cells in primary culture. These studies were then extended to analyze changes in the four cytochrome P450 mRNA levels and the production of cortisol in adrenocortical cells isolated from halothane-sensitive (HAL-S) and halothane-resistant (HAL-R) pigs.

MATERIALS AND METHODS

Materials

Porcine ACTH, 8-bromo cAMP (8-Br-cAMP), insulin, human transferrin, collagenase type I (300 U/mg), DNAase I (from bovine pancreas), bovine serum albumin (BSA), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Recombinant bovine fibroblast growth factor (bFGF) was obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). Ham's F12/Dulbecco's modified Eagle's medium (F12/DMEM; 1:1), horse serum, fetal calf serum, and other culture supplies were purchased from Gibco-BRL (Grand Island, NY).

Selection of HAL-S and HAL-R Pigs

Sixteen male Lanyu small-ear miniature pigs (9 HAL-R, 7 HAL-S) from seven litters, born at the Taiwan Livestock Research Institute Taitung Animal Propagation Station, were studied. All animals received care according to the guidelines of the Animal Care Committee of the National Science Council, Taiwan. The pigs were selected based on the presence of muscle rigidity during halothane anesthesia at the age of 12–16 weeks. Pigs developing muscle rigidity during tests were classified as stress-susceptible (*nn*) or HAL-S pigs, whereas those remaining relaxed were classified as stress-resistant (*NN* or *Nn*) or HAL-R pigs. In this study, HAL-R and HAL-S animals were obtained within each litter for one experiment. Halothane genotypes of HAL-R pigs were not determined. The HAL-R and HAL-S pigs were slaughtered and adrenal glands were removed for preparation of adrenocortical cells. In some experiments, adrenal glands were collected from prepubertal pigs of unspecified age and sex at a local slaughterhouse.

Preparation and Culture of Pig Adrenocortical Cells

Adrenal glands collected from freshly slaughtered pigs were carried to the laboratory in chilled HEPES buffer (137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 25 mM HEPES, 10 mM glucose and 360 μM $CaCl_2$) containing 0.1% BSA, penicillin (100 U/ml), and streptomycin sulfate (200 μg/ml). Cells were prepared as described previously [Gospodarowicz et al., 1977] with some modifications. The adrenal glands were trimmed free of fat and connective tissue and cut in half by a longitudinal incision. The medulla was removed and the cortex was scraped off the capsule, minced into small pieces, and washed four times with HEPES-0.1% BSA buffer. The adrenal pieces were dispersed by constant stirring for 60 min at 37°C in HEPES-0.1% BSA buffer containing 0.2% collagenase and 0.01% DNAase. No attempt was made to separate the zona glomerulosa from the zona fasciculata and zona reticularis. The cells were filtered through two layers of cheesecloth, then washed three times with HEPES-0.1% BSA buffer, collected by centrifugation for 5 min at 200*g*, resuspended in F12/DMEM supplemented with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), insulin (10 μg/ml), human transferrin (5 μg/ml), and bFGF (0.1 ng/ml) in the presence of 12.5% horse serum and 2.5% fetal calf serum, and plated onto 60-mm (for cortisol and cAMP determinations) or 100-mm (for RNA isolation) tissue culture dishes at a density of $\sim 10 \times 10^6$ cells/4 ml (60-mm dishes) or 40×10^6 cells/6 ml (100-mm dishes). The cell system was incubated in duplicate at 37°C in a 5% CO_2–air atmosphere for 6 days with a medium change 3 days after the cells were prepared. Before being used in an experiment, the cell cultures were rinsed twice with phosphate-buffered saline (PBS). Cells were then incubated in 2 ml (60-mm dishes) or 4 ml (100-mm dishes) F12/DMEM (without serum, insulin, transferrin, and bFGF supplement) containing test substances for the periods as indicated in the Results section. The supernatants were removed and kept frozen at −20°C until assayed. A single preliminary experiment was conducted to determine the optimal duration of culture for ACTH stimulation on cells every 24 h after the cell seeding up to 10 days. After 2 days of culture, the cells began to have a cortisol response to 18-h exposure to ACTH (10^{-8} M), and a maximal stimulatory effect was reached on day 5 or day 6. The experiments were, therefore, carried out using cells on day 6 of culture. Data reported in Figures 1 and 2 represented a duplicate determination of a typical experiment performed three times. In this part of study, data were obtained from different preparations of cells, each being prepared from 10–12 adrenal glands. Within each preparation, there were two replicates per treatment. Data presented in Figures 3 and 6 were ob-

tained from four and three experiments, respectively. Within each experiment, cells from litter-matched HAL-R and HAL-S (one of each) pigs were prepared separately. In one of the four experiments, three HAL-R and one HAL-S pigs were used. Data presented in Figure 4 were obtained from four sets of cells prepared from litter-matched HAL-R and HAL-S pigs used for cultures in Figure 3.

RNA Isolation and Northern Blotting

Total RNA was recovered by phenolic extraction in the presence of guanidinium thiocyanate, using a simplified version of a previously described method by Chomczynski and Sacchi [1987]. Cells on 100-mm culture dishes were washed in cold PBS solution before recovery (at room temperature) into 2 ml TRISOLV solution (Cinna Biotecx, Houston, TX) and transfer to two microfuge tubes (1 ml/tube). Phase separation was then achieved by the addition of 0.2 ml chloroform per 1 ml of the TRISOLV, standing on ice for 2 min, and centrifugation (12,000*g*; 15 min; 4°C). The upper phase (0.7 ml) was transferred to a second microfuge tube, and RNA was precipitated by the addition of 0.7 ml isopropanol per 1 ml of the TRISOLV used for homogenization and standing on ice for 10 min. RNA was recovered by centrifugation (12,000*g*; 10 min; 4°C) and washed once in 100% ethanol (0.5 ml). The RNA pellet was then stored in 100% ethanol (0.5 ml) at −85°C until Northern blot analysis. Before analysis, samples were centrifuged for 5 min at 12,000*g* at 4°C. The pellet was resuspended in 75% ethanol (1 ml), then reprecipitated by centrifugation (12,000*g*; 5 min; 4°C). The RNA pellet was dissolved in 6–10 μl of Milli Q water and quantitated by reading the absorbance at 260 nm.

The same amount of RNA sample (25 μg) was denatured in a mixture of glyoxal (1 M) and sodium phosphate (10 mM; pH 6.5) for 60 min at 50°C, then size-fractionated by electrophoresis gels containing 1% agarose using sodium phosphate (10 mM; pH 6.5) as the running buffer and transferred to Hybond-N nylon membranes (Amersham International plc., Amersham, Bucks, UK) by pressure blotting (50–60 mbar; 90 min; VacuGene XL Blotting Unit, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) [Thomas, 1980]. RNA was crosslinked to nylon membrane with UV light using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The migration position of ribosomal RNA (28S and 18S) was determined by 0.02% methylene blue (in 0.5 M sodium acetate; pH 4.8) staining.

cDNA Labeling and Hybridization Analysis

Human P450scc, $P450_{17\alpha}$, and $P450_{c21}$, bovine $P450_{11\beta}$, and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were used as probes. $P450_{scc}$, $P450_{17\alpha}$, and $P450_{c21}$ cDNAs were gifts from Dr. Bon-Chu Chung (Institute of Molecular Biology, Academia Sinica, Nankang, Taiwan). $P450_{11\beta}$ cDNA was provided by Dr. M. R. Waterman (Department of Biochemistry, Vanderbilt University, Nashville, TN). Mouse GAPDH cDNA was a gift from Dr. Hsiao-Sheng Liu (Department of Microbiology, National Cheng Kung University Medical College, Tainan, Taiwan). Plasmid preparation of the five different cDNA inserts was carried out using Qiagen anion-exchange columns (Qiagen protocol; DIagen GmbH, Dusseldorf, Germany). The cDNA inserts were removed from the plasmids by digestion with restriction endonucleases. The respective cDNA inserts were purified by electrophoresis in 1% agarose gel after digestion with appropriate restriction enzymes. The specific P450 cDNAs were labeled with [α-^{32}P]deoxy-CTP (specific activity = 3,000 Ci/mmol; Amersham) to a specific activity of 10^8–10^9 c.p.m./μg using the Megaprime DNA Labelling System (Amersham) [Feinberg and Vogelstein, 1983]. The labeled cDNAs were separated from free radionucleotides by Sephadex G-50 (Fine; Pharmacia LKB Biotechnology AB) chromatography.

The membranes were prehybridized (1 h) at 60°C with shaking in QuikHyb solution (Stratagene). Hybridization was carried out for 3 h at 60°C in the same solution containing heat-denatured [^{32}P]-labeled P450 cDNAs and salmon sperm DNA (10 mg/ml) on the same membrane. Thereafter, membranes were washed twice in 2x sodium chloride-sodium citrate (SSC; 1x SSC contains 15 mmol sodium citrate/l and 150 mmol sodium chloride/l)/0.1% (wt/vol) sodium dodecylsulphate (SDS) at room temperature, for 15 min each, and once in 0.1x SSC-0.1% SDS at 42°C for 30 min by shaking. Finally, the membranes were exposed to a Fuji medical X-ray film at −85°C using an intensifying screen. The film was subsequently developed using conventional procedures. When the

membranes were used for rehybridization with other probes, preexisting [^{32}P]-labeled probe on the membranes was removed by boiling the membranes in 0.1x SSC–0.1% SDS until background counts were less than 5 cps. A final rehybridization was carried out using a mouse GAPDH cDNA probe to control for variation in gel loading. Hybridization intensities of each specific mRNA were quantified using a densitometric system (Bio-Rad Video Densitometer, model 620; Bio-Rad Laboratories, Richmond, CA). Results are expressed as arbitrary units of $P450_{scc}$, $P450_{17_{17\alpha}}$, $P450_{c21}$, or $P450_{11\beta}$/GAPDH mRNA ratios.

Radioimmunoassay of Cortisol and cAMP

A commercially available radioimmunoassay (RIA) kit purchased from Amersham was used to measure medium concentration of cAMP. The detection limit was 1 pmole/assay tube.

Cortisol concentration in the unextracted medium was measured by RIA according to Li and Wagner [1983]. The antiserum to cortisol-3-BSA was obtained from NBL Biochemical Industry Corp. (Taichung, Taiwan) and was used at the dilution of 1:60,000 in 0.01 M Tris buffer (pH 7.4). The cross-reactivity of this antiserum was 4.9% for corticosterone, 4.0% for testosterone, 0.9% for progesterone, and <0.01% for androstenediol, androstenedione, 17α- and 17β-estradiol, pregnenolone, and 17α-hydroxyprogesterone. The detection limit was 3.12 pg per assay tube. The intraassay variation, determined by duplicates of three dose levels of the control medium from the adrenocortical cell pool, was <10%.

Statistical Analysis

Experimental data are presented as the mean ± SEM of measurements from duplicate culture dishes from a typical experiment unless otherwise indicated. Cortisol production was expressed as nanograms per 10^6 cells with cell numbers at the beginning of the experiment used for this calculation. Cortisol data obtained from the cells that were isolated from prepubertal gilts were analyzed by least-squares analysis of variance (ANOVA), with dose of ACTH or 8-Br-cAMP and time of incubation with ACTH or 8-Br-cAMP as main effects. Additionally, data were subjected to linear regression analysis to evaluate change in cortisol production over dose of ACTH or 8-Br-cAMP at each period. The data of the responses of cells isolated from HAL-S and HAL-R pigs to ACTH or 8-Br-cAMP were analyzed using the Allfit program. The differences between two curves were determined by comparison of their net areas. Mean levels of mRNA for $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, or $P450_{11\beta}$ were expressed as relative level of mRNA/GAPDH mRNA ± SEM. These values were analyzed by one-way ANOVA. Duncan's multiple range test was used to make comparison among the means if the ANOVA proved significant. Two means were compared using Student's *t*-test.

RESULTS

Time–Dose Dependency of Adrenocortical Cell Cortisol and cAMP Production in Response to ACTH Stimulation

Effects of varying time of exposure and concentration of ACTH on cortisol and cAMP concentrations in medium are shown in Figure 1. In the absence of ACTH, cortisol accumulated in medium during 24 h in the primary 6-day cultured cells, and this increase was linear ($P < 0.001$), i.e., rate of accumulation increased with time (Fig. 1A). Within time periods, increase in cortisol production by ACTH over the range 10^{-10}–10^{-6} M was linear ($P < 0.001$). Dose–response slopes were not different when exposure was for 18 or 24 h. Stimulation with 10^{-10} M ACTH had no effect on cortisol production for up to 18 h. The increase in cortisol production over controls, stimulated by 10^{-9} M to 10^{-6} M of ACTH, increased at least 2.8-fold at each period. A maximal effect on cortisol production was reached with 10^{-8} M ACTH. At 24 h, this stimulation produced a cortisol production of 30.59 ± 0.53 ng/10^6 cells. The basal rate of cortisol production increased from 0.24 ng/10^6 cells after 1 h of incubation to 8.47 ng/10^6 cells after 24 h (Fig. 1A).

Similar to cortisol production, there was a linear ($P < 0.05$) increase in the concentration of cAMP in media during 24 h of ACTH stimulation (Fig. 1B). There was no difference between dose–response slopes with exposure for 18 or 24 h. In the absence of ACTH, the medium concentration of cAMP was undetectable for up to 6 h. Stimulation with 10^{-10} M ACTH had no effect on cAMP production at each period. At 1 h, stimulation with 10^{-8} M ACTH also had no effect on cAMP concentration, whereas ACTH at the concentrations of 10^{-8},

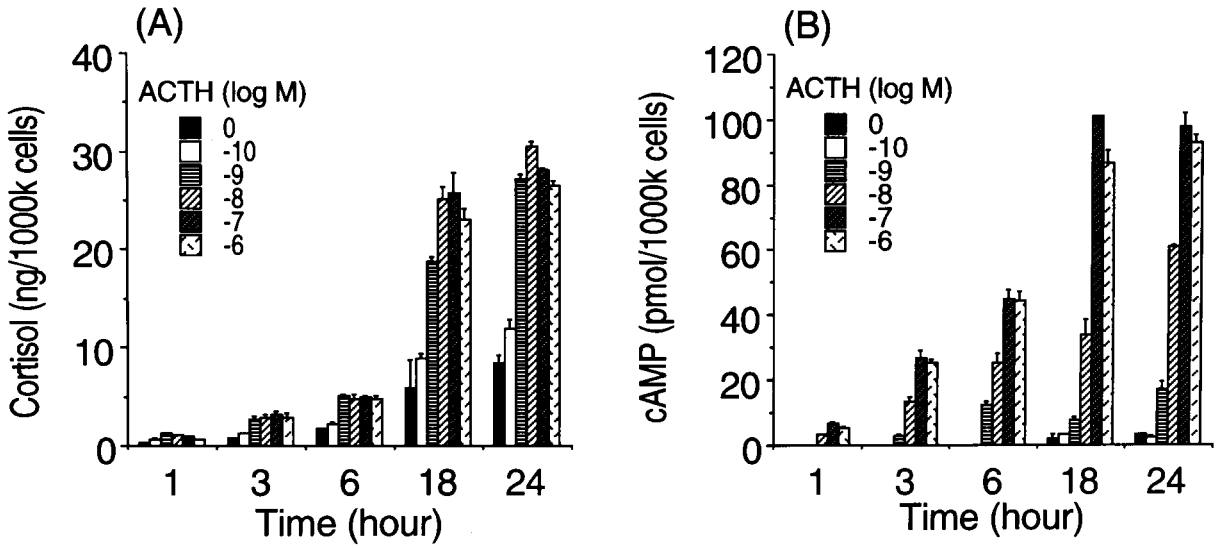

Fig. 1. Time–dose-dependency of the effect of ACTH on pig adrenocortical cell cortisol (ng/dish) and cAMP (pmole/dish) production. Cortisol and cAMP production was normalized per 10^6 cells. Adrenal glands were obtained from prepubertal pigs of unspecified age and sex at a local slaughterhouse. On day 6 of the culture, cells were washed twice with phosphate-buffered saline and then incubated for 1–24 h with increasing concentrations (10^{-10}– 10^{-6} M) of ACTH in Ham's F12/ Dulbecco's modified Eagle's medium without serum and insulin, transferrin, and bovine fibroblast growth factor supplement. The concentrations of cortisol (**A**) and cAMP (**B**) were measured in the incubation media by radioimmunoassay. Results are the mean ± SEM of duplicate cultures. SEM is not shown when too small to be graphed legibly. Comparable results were obtained in two other sets of experiments.

10^{-7}, and 10^{-6} increased cAMP production in a dose-dependent manner. The increase in cAMP concentration over controls, induced by 10^{-9} M–10^{-6} M ACTH, increased from 3- to 25.2-fold, 12.4- to 44.3-fold, 3.7- to 40.7-fold, and 5.2- to 28.1-fold at incubation periods of 3, 6, 18, and 24 h, respectively.

Time–Dose Dependency of Adrenocortical Cell Cortisol Production in Response to 8-Br-cAMPStimulation

Effects of varying time of exposure and concentration of 8-Br-cAMP on cortisol production in medium are shown in Figure 2. 8-Br-cAMP increased cortisol production relative to controls when present for 1, 3, 6, 18, or 24 h. The magnitude of cortisol production, within these periods, was linearly ($P < 0.05$) related to concentrations of 8-Br-cAMP used. A maximal effect on cortisol production was reached with 1 mM 8-Br-cAMP. This stimulation produced a cortisol production of 0.27 ± 0.02, 0.72 ± 0.03, 2.62 ± 0.30, 4.64 ± 0.15, and 4.4 ± 0.14 ng/10^6 cells after 1, 3, 6, 18, and 24 h of incubation, respectively.

Effect of ACTH or 8-Br-cAMP on Adrenocortical Cell Cortisol Production in HAL-S and HAL-R Pigs

The addition of ACTH to primary cell cultures increased cortisol production in a dose-related manner in both HAL-R and HAL-S pigs, as shown in Figure 3A. HAL-S pigs had 85% lower basal cortisol production and 88% lower maximal cortisol production stimulated by 10^{-6} M ACTH compared to HAL-R pigs ($P < 0.05$). The net area under the two curves was significantly ($P < 0.001$) different (Fig. 3A). There were no differences in EC_{50} values (HAL-S, 0.17 nM; HAL-R, 0.19 nM) between cell populations isolated from HAL-S and HAL-R pigs.

However, the percent cortisol production above baseline at any single dosage was the same throughout the cortisol dose–response curve in HAL-S compared to HAL-R pigs (Fig. 3B). There were no differences in the net area under the two curves.

As shown in Figure 4A, both HAL-R and HAL-S pig adrenocortical cells responded in a dose-related manner to increasing concentrations of the cAMP analog 8-Br-cAMP ($P <$

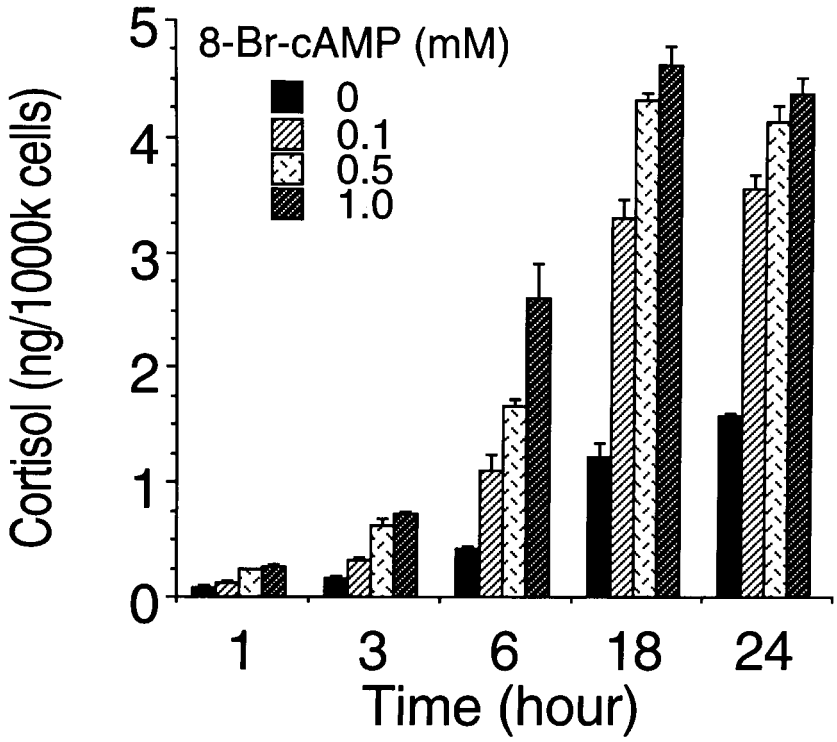

Fig. 2. Time–dose dependency of the effect of 8-bromo-cyclic AMP (8-Br-cAMP) on the production (ng/dish) of cortisol from pig adrenocortical cells in culture. Cortisol production was normalized per 10^6 cells. Adrenal glands were obtained from prepubertal pigs of unspecified age and sex at a local slaughterhouse. On day 6 of the culture, cells were washed twice with phosphate-buffered saline and then incubated for 1–24 h with increasing concentrations (0.1–1 mM) of 8-Br-cAMP in Ham's F12/ Dulbecco's modified Eagle's medium without serum and insulin, transferrin, and bovine fibroblast growth factor supplement. The concentrations of cortisol were measured in the incubation media by radioimmunoassay. Results are the mean ± SEM of duplicate cultures. SEM is not shown when too small to be graphed legibly. Comparable results were obtained in two other sets of experiments.

0.05). HAL-S pigs had significantly lower ($P < 0.05$) cortisol production stimulated by 0.1, 0.5, and 1.0 mM 8-Br-cAMP compared to HAL-R pigs. The net area under the two curves was significantly ($P < 0.05$) different (Fig. 4A). However, the percent cortisol production above baseline at every dose of 8-Br-cAMP tested was not different (Fig. 4B). The two curves are not different as evaluated by comparison of their net area.

Time–Course and Dose–Response of 8-Br-cAMP Effects on $P450_{scc}$, $P450_{17_\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNA Levels

To learn whether the changes observed in the steroidogenesis of HAL-S pig adrenocortical cells after treatment with ACTH or 8-Br-cAMP were caused by changes in the expression of the genes encoding specific adrenal functions, we determined and compared the relative levels of the four steroid hydroxylase mRNAs. In the first series of experiments, we investigated the changes in $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNA levels in cells obtained from prepubertal pig adrenal glands by enzymatic dispersion. Figure 5 shows the time–course and dose–response curve of 8-Br-cAMP on the four P450 mRNAs. Under basal conditions, pig adrenocortical cells had a defined basal level of mRNA that hybridized to the specific $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ cDNA probes (Fig. 5A,B). After an 18-h treatment, increasing doses of 8-Br-cAMP (0.1–1 mM) produced a significant ($P < 0.05$) mRNA accumulation, reaching a maximal effect at a concentration of 0.5 mM. At this concentration, the accumulation of $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNA represents a 2.6-, 8.6-, 6.7-, and 8.7-fold increase over control values, respectively. Increasing periods of incubation times with 8-Br-cAMP (0.5 mM) produced a progressive accumulation of all four mRNAs. Accordingly, an 18-h treatment with 0.5 mM 8-Br-cAMP was used in subsequent studies.

Figure 6A shows the expression of the four genes in adrenocortical cells isolated from HAL-S and HAL-R pigs and treated with 0.5 mM 8-Br-cAMP for 18 h. In the absence of 8-Br-cAMP, the four genes were expressed constitutively in both HAL-S and HAL-R pig adrenal cells. Densitometric scanning of the autoradiograph shown in Figure 6B indicated that the relative amounts of $P450_{scc}$ and $P450_{17\alpha}$ mRNAs in HAL-S pig adrenal cells were between 48 and 53% of those detected in

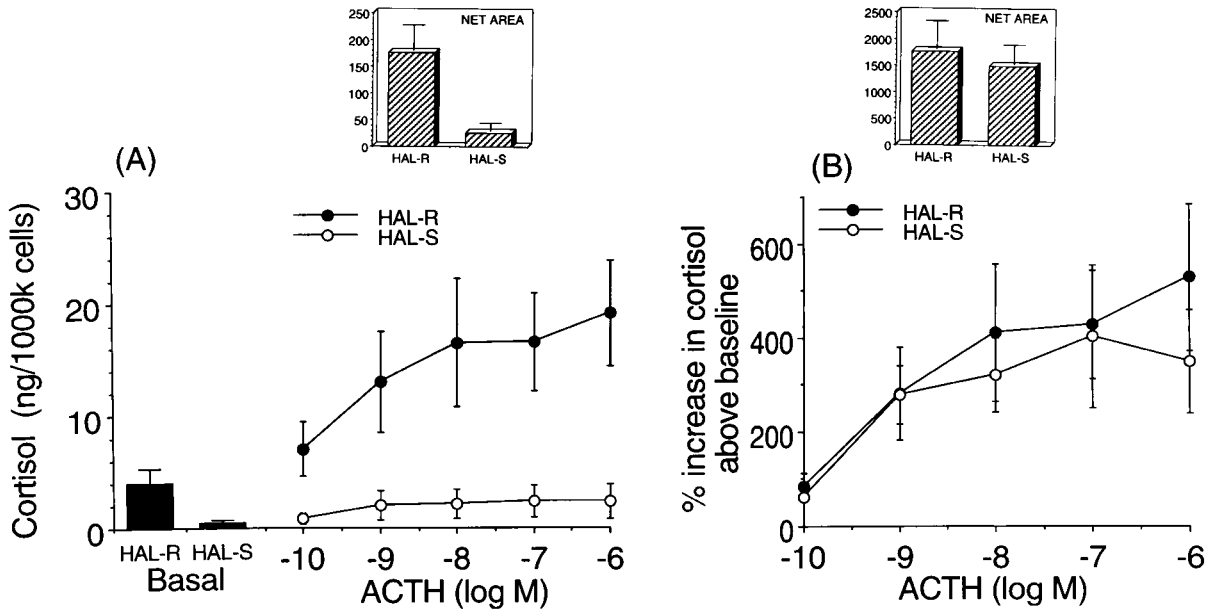

Fig. 3. **A:** Dose–response data for ACTH-induced cortisol production (ng/dish) in halothane-resistant (HAL-R) and halothane-sensitive (HAL-S) pig adrenocortical cell cultures. Cortisol production was normalized per 10^6 cells. Six-day cell cultures were incubated with various concentrations of ACTH in serum-free medium without insulin, transferrin, and bovine fibroblast growth factor supplement. After 24 h, media from individual dishes were collected and cortisol was evaluated by radioimmunoassay. **B:** Percent of stimulation above baseline. Each point represents the mean ± SEM from four independent experiments. Within each experiment, adrenocortical cells isolated from litter-matched HAL-R and HAL-S (one of each) pigs were prepared separately. Within each cell preparation, there were two replicates per treatment. In one of the four experiments, three HAL-R and one HAL-S pigs were used. The net area under each curve is shown in the inset above (A) and (B).

HAL-R pig adrenal cells ($P < 0.05$). No difference in the amounts of $P450_{c21}$ and $P450_{11ß}$ was seen in HAL-S and HAL-R pig adrenal cells. Addition of 8-Br-cAMP at a concentration of 0.5 mM resulted in a uniform increase in the levels of all four P450 mRNAs (Fig. 6A,B) in both HAL-S and HAL-R pig adrenal cells. However, the amounts of $P450_{scc}$ mRNA in HAL-S pig adrenal cells were 67% ($P < 0.05$) of those measured in HAL-R pig adrenal cells, whereas the amounts of $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNAs were similar in these cells.

DISCUSSION

Data presented in this study demonstrate that although the absolute HAL-S cortisol responses to various doses of ACTH were 87% lower than in HAL-R pigs, when corrected for the lower baseline, the percent HAL-S adrenocortical cell cortisol production at every ACTH dose was the same as the corresponding HAL-R responses. The EC_{50} for ACTH-induced cortisol production was 0.17 and 0.19 nM in HAL-S and HAL-R pigs, respectively. This would indicate that the lower absolute amount of cortisol produced by the HAL-S adrenocortical cells cannot be explained by a defective receptor. Our data also suggest that the altered pituitary–adrenal function in HAL-S pigs probably is not related to a cAMP defect at the adrenal level because 8-Br-cAMP stimulated HAL-S and HAL-R adrenocortical cell cultures almost equally in a dose-related manner when corrected for differences in basal secretion. Thus, the differences in basal and ACTH-stimulated cortisol production between HAL-S and HAL-R pigs in vitro appeared to be related to the differences in basal cortisol content rather than to the differences in responsiveness to ACTH or cAMP.

The 85% greater basal production of cortisol in HAL-R cultures was consistent with the larger amount of $P450_{scc}$ (47% greater in HAL-R than HAL-S) and $P450_{17_\alpha}$ (52% greater in HAL-R than HAL-S) mRNAs measured by Northern analysis. The larger amount of $P450_{scc}$ and $P450_{17\alpha}$ mRNAs in HAL-R pigs was not caused by greater adrenocortical cell mass because of similar adrenal cortex weight (0.51 ± 0.08 g for HAL-R compared to 0.65 ± 0.12 g for HAL-S). Thus, these findings suggest

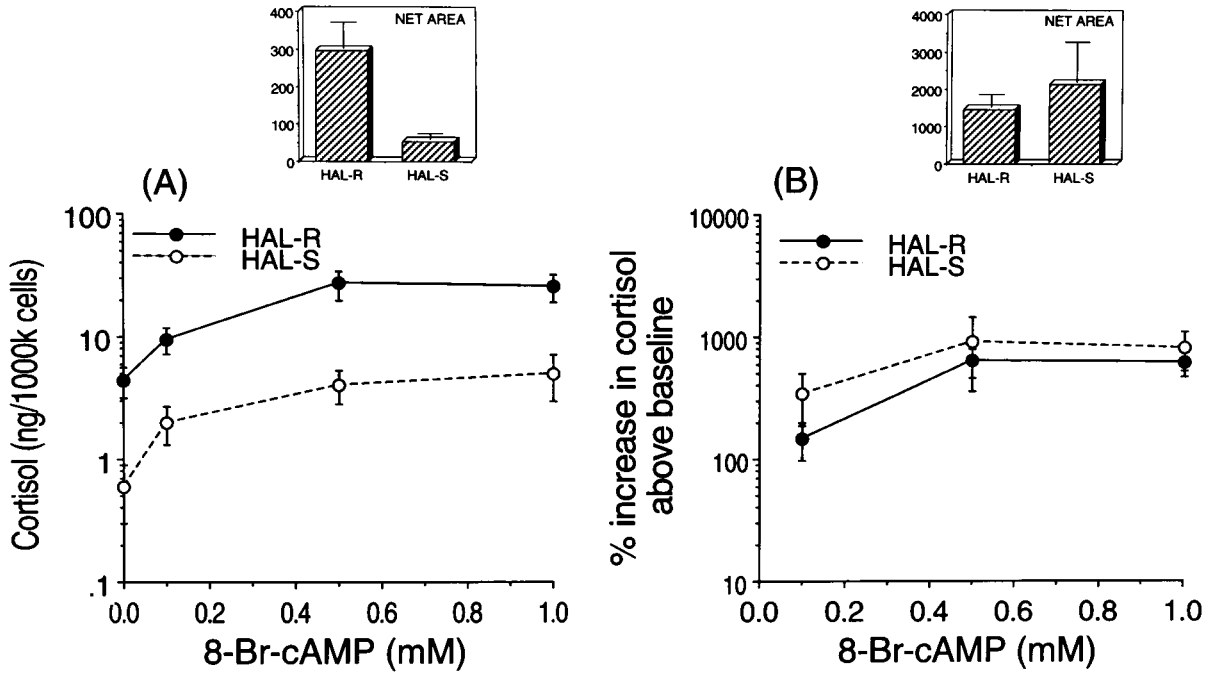

Fig. 4. **A:** Dose–response data for 8-bromo-cyclic AMP (8-Br-cAMP)-induced cortisol production (ng/dish) in halothane-resistant (HAL-R) and halothane-sensitive (HAL-S) pig adrenocortical cell cultures. Cortisol production was normalized per 10^6 cells. Six-day cell cultures were incubated with various concentrations of ACTH in serum-free medium without insulin, transferrin, and bovine fibroblast growth factor supplement. After 24 h, media from individual dishes were collected and cortisol was evaluated by radioimmunoassay. **B:** Percent of stimulation above baseline. Each point represents the mean ± SEM from four independent experiments. Cells prepared from litter-matched HAL-R and HAL-S pigs used for cultures in Figure 3 were used in this study (see legend to Fig. 3). The net area under each curve is shown in the inset above (A) and (B). Note logarithmic scale on ordinate for cortisol concentration and percent increase in cortisol above baseline in (A) and (B), respectively.

that the lower baseline cortisol production in cell cultures from HAL-S pigs may be, in part, caused by a decrease in $P450_{scc}$ and $P450_{17\alpha}$ mRNA expression. The decrease may represent a primary genetic feature of HAL-S pigs. The possibility that the steroidogenic enzyme content and its activity in HAL-S cells may be reduced remains to be investigated. Another possibility is that the cholesterol content in HAL-S cells may be reduced. Moreover, the steroidogenic acute regulatory (StAR) protein has recently been shown to be a factor necessary for cholesterol transport into adrenal and gonadal mitochondria, which is the regulated rate-limiting step in steroidogenesis [Stocco and Clark, 1996]. Whether the StAR protein level in HAL-S cells is decreased also needs to be examined.

The association between the *Hal* gene that confers stress susceptibility and adrenocortical activity was the focus of this study. The anesthetic most commonly associated with malignant hyperthermia is halothane, and this agent has been used effectively in Lanyu small-ear miniature pigs, a local breed in Taiwan, for attempts to eliminate the *Hal* gene from breeding stock. The frequency of the *n* allele in Lanyu pigs was estimated to be ~0.5 (data not shown). Because the halothane challenge test detects the *nn* genotype pigs but does not differentiate between *NN* and *Nn* genotype pigs [Webb, 1980], we do not know the genotypes (normal, *NN*; heterozygotes, *Nn*) of HAL-R pigs selected for use in this study. The DNA-based assay for a C-T mutation at base pair 1,843 of the skeletal muscle ryanodine receptor cDNA, which is very highly correlated with porcine stress syndrome, has not yet been performed in pigs bred for our studies. However, our present in vitro work corroborates and extends the in vivo work of others: stress-susceptible pigs (halothane tested) had a lower resting cortisol concentration [Mitchell and Heffron, 1981]. Before transport, the homozygous halothane-

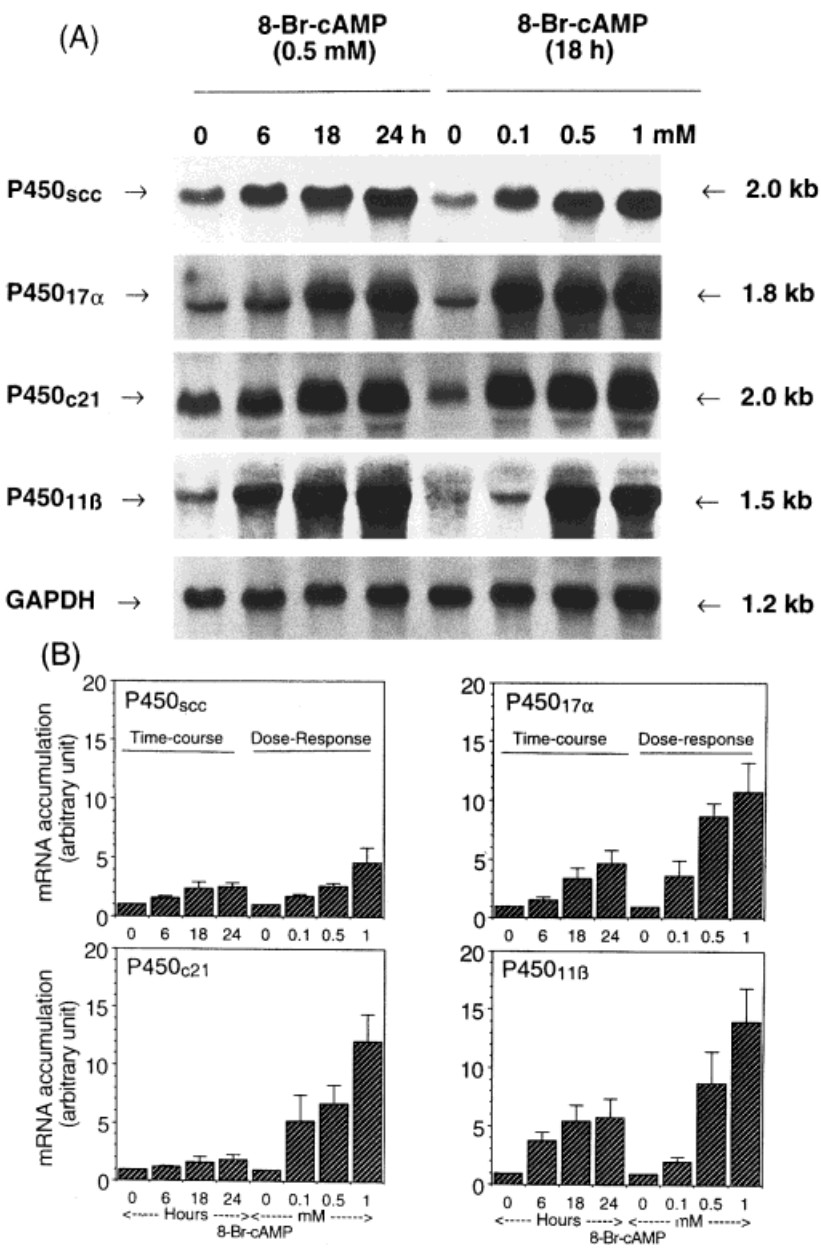

Fig. 5. Time course and dose–response effects of 8-bromo-cyclic AMP (8-Br-cAMP) on the accumulation of mRNAs encoding $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$. Adrenal glands were obtained from prepubertal pigs of unspecified age and sex at a local slaughterhouse. On day 6 of the culture, adrenocortical cells were washed twice with phosphate-buffered saline and then incubated for 6–24 h with 8-Br-cAMP (0.1, 0.5, and 1 mM) in Ham's F12/Dulbecco's modified Eagle's medium without serum. Total RNA was isolated and 25 μg from each sample were size fractionated by 1% agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with [^{32}P]deoxy-CTP-labeled cDNA encoding human $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and bovine $P450_{11\beta}$. Hybridization obtained with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a loading control. **A:** Representative Northern blots. **B:** Autoradiographs were quantitated by densitometry; data were corrected for the amount of GAPDH in each lane and normalized to the value of untreated cultures that were given the arbitrary value 1. The results are expressed as mean ± SEM from four independent experiments.

positive *nn* pigs had a lower plasma cortisol content [Geers et al., 1994]. After heat stress and exercise, stress-susceptible pigs had a lower net increase in cortisol [Mitchell and Heffron, 1982; Nyberg et al., 1988]. Such stress-susceptible pigs were also shown to have higher levels of circulating ACTH without a corresponding increase in plasma adrenocorticoids [Marple et al., 1972]. In contrast to these results, Marple et al. [1974] found a higher cortisol concentration in pigs from herds bred to be stress susceptible. The lack of correlation between stress susceptibility and adrenal response to ACTH or stressors was also observed in crossbred pigs [von Borell and Ladewig, 1989]. Moreover, our work builds on this information by demonstrating alterations in adrenocortical function at the cellular level in a genetic-dependent, stress-response model.

There is limited information on the regulation of $P450_{scc}$, $P450_{17_\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNAs in pig adrenocortical cells. In this study, we analyzed a time–course and dose–response effects of 8-Br-cAMP on the modulation of $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNA levels. We showed that, in pig adreno-

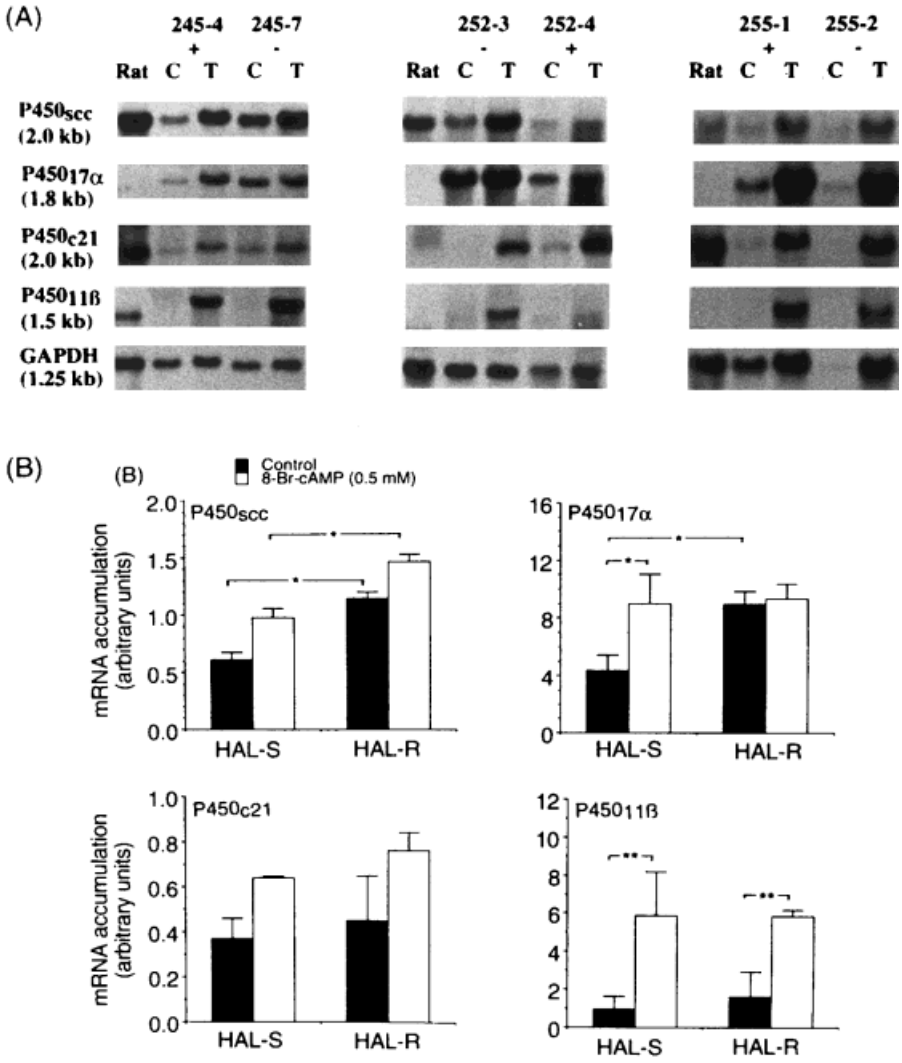

Fig. 6. Expression of cytochrome P450 mRNAs encoding $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ in adrenocortical cells isolated from halothane-sensitive (HAL-S) and halothane-resistant (HAL-R) pigs. Six-day cell cultures were incubated with or without 8-bromo-cyclic AMP (8-Br-cAMP) (0.5 mM) for 18 h in Ham's F12/ Dulbecco's modified Eagle's medium without serum. Total RNA was isolated and 25 μg from each sample were size fractionated by 1% agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with [^{32}P]deoxy-CTP-labeled cDNA encoding human $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and bovine $P450_{11\beta}$. Hybridization obtained with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA was used as a loading control. **A:** Representative Northern blots; C, control; T, 8-Br-cAMP-treated; pigs 245-4 and 245-7, 252-3 and 252-4, and 255-1 and 255-2 were from three different litters. Total RNA extracted from rat adrenal glands was used as positive control. **B:** Autoradiographs were quantitated by densitometry; data were corrected for the amount of GAPDH in each lane. Results are the mean ± SEM of P450s/GAPDH mRNA ratios. $^*P < 0.05$, $^{**}P < 0.01$.

cortical cells, the effects of 8-Br-cAMP on the levels of all four P450 mRNAs are dose dependent and time dependent, and are therefore qualitatively similar to those observed for $P450_{scc}$ [John et al., 1984], $P450_{17\alpha}$ [Zuber et al., 1986], $P450_{c21}$ [John et al., 1986a] and $P450_{11\beta}$ [John et al., 1985] in bovine adrenal cells. Increased levels of RNAs encoding $P450_{scc}$ [DiBlasio et al., 1987; John et al., 1986b] and $P450_{17\alpha}$ [DiBlasio et al., 1987] were also observed in human fetal adrenal cells stimulated with cAMP. In the present study, we also demonstrated that cortisol production was stimulated by both ACTH treatment protocols and 8-Br-cAMP in a similar pattern. Cyclic AMP production also was stimulated by ACTH in a time- and dose-related manner. Thus, our results confirm the stimulatory ef-

fects of ACTH or cAMP on the production of cortisol and expression of steroid hydroxylase mRNA levels in the adrenal cortex. Our results also provide evidence that the cAMP pathway mediates the effect of ACTH on steroidogenic enzymes in the pig adrenocortical cells.

In the present study, it took pig adrenocortical cells 4-6 days to develop a confluent monolayer after plating. During this time, the cultures were maintained in the presence of serum but in the absence of ACTH. The mRNA for $P450_{scc}$, $P450_{17_\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ remained at lowered but readily detectable levels in such unstimulated cultures by day 6. After culturing in serum-free medium for 18 h, the amount of $P450_{scc}$ mRNA remained at about the same low level, whereas the amounts of $P450_{17_\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ mRNAs declined to 16.8, 8.5, and 6%, respectively, of the value by day 6. Thus, in this report, the behavior of cultured pig adrenocortical cells was similar to that of cultured bovine [Güse-Behling et al., 1992], ovine [Rainey et al., 1990], and human fetal and adult adrenal cells [DiBlasio et al., 1987; Ilvesmaki and Voutilainen, 1991]. Our data also suggest that regulation of $P450_{scc}$, $P450_{17\alpha}$, $P450_{c21}$, and $P450_{11\beta}$ gene expression in pig adrenal cortex involves a cAMP-independent mechanism.

In summary, we have provided evidence that an HPA axis defect in HAL-S pigs is at the adrenal level. This defect appears to be at the level of $P450_{scc}$ gene expression. The genetic disorder influencing the amount of cholesterol side-chain cleavage cytochrome P450 could be partially related to reduced cortisol production by ACTH stimulation. Whether there are differences in the expression of StAR protein needs to be evaluated.

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