Inhibition of Adrenal Cortical Steroid Formation by Procaine Is Mediated by Reduction of the cAMP-Induced 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Messenger Ribonucleic Acid Levels

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ABSTRACT

Elevated glucocorticoid levels are associated with many diseases, including age-related depression, hypertension, Alzheimer’s disease, and acquired immunodeficiency syndrome. Cortisol-lowering agents could provide useful complementary therapy for these disorders. We examined the effect of procaine and procaine in a pharmaceutical formulation on adrenal cortical steroid formation. Procaine inhibited dibutyryl cyclic AMP (dbcAMP)-induced corticosteroid synthesis by murine Y1 and human H295R adrenal cells in a dose-dependent manner without affecting basal steroid formation. Treatment of rats with the procaine-based formulation reduced circulating corticosterone levels. This steroidogenesis-inhibiting activity of procaine was not observed in Leydig cells, suggesting that the effect was specific to adrenocortical cells. In search of the mechanism underlying this inhibitory effect on cAMP-induced corticosteroidogenesis, procaine was found to affect neither the cAMP-dependent protein kinase activity nor key proteins involved in cholesterol transport into mitochondria, cytochrome P450 side chain cleavage enzyme expression, and enzymatic activities associated with cholesterol metabolism to final steroid products. However, procaine reduced in a dose-dependent manner the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) activity and the dbcAMP-induced HMG-CoA reductase mRNA levels by affecting mRNA stability. These data suggest that the inhibitory effect of procaine on cAMP-induced corticosteroid formation is due to the reduced synthesis of cholesterol. This modulatory effect of procaine on HMG-CoA reductase mRNA expression was also seen in dbcAMP-stimulated Hepa1-6 mouse liver hepatoma cells. Taken together, these results suggest that procaine may provide a pharmacological means for the control of hormone-induced HMG-CoA reductase mRNA expression and hypercortisolemia.

Cortisol is the main circulating glucocorticoid in humans and it is involved in different physiological functions such as sleep cycle regulation, metabolism, immunity, mood normalization, memorization, and learning. Excessive cortisol synthesis leads to changes in metabolism, cognitive impairment (McEwen, 1994), and immunosuppression (Chrousos and Gold, 1992). Indeed, abnormalities at different levels of the hypothalamic-pituitary-adrenal (HPA) axis have been reported in several diseases such as psychiatric disorders, including depression and mood alteration (Kiraly et al., 1997; Tafet et al., 2001); acquired immunodeficiency syndrome (AIDS) (Corley, 1996; Bhansali et al., 2000; Christeff et al., 2000); multiple sclerosis (Erkut et al., 2002); dementia (Maeda et al., 1991; Polleri et al., 2002), including Alzheimer’s disease (AD) (Swaab et al., 1994; O’Brien et al., 1996; Weiner et al., 1997; Giubilei et al., 2001; Rasmuson et al., 2002); and breast cancer outcome (Luecken and Compas, 2002). It has been proposed that disruption of hormonal balance in these diseases leads to increased cortisol production, resulting in elevated concentrations of cortisol in cerebrospinal fluid (Swaab et al., 1994; Erkut et al., 2002), blood (Weiner et al., 1997; Bhansali et al., 2000; Rasmuson et al., 2002), urine (Maeda et al., 1991), and saliva (Giubilei et al., 2001).

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ABBREVIATIONS: HPA, hypothalamus-pituitary-adrenal; AIDS, acquired immunodeficiency syndrome; AD, Alzheimer’s disease; P450scc, cytochrome P450 side chain cleavage; PKA, cAMP-dependent protein kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; dbcAMP, dibutyryl cyclic AMP; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; StAR, steroidogenesis acute regulatory protein; PCR, polymerase chain reaction; Q-PCR, real-time quantitative PCR; ANOVA, analysis of variance; PBR, peripheral-type benzodiazepine receptor.
As every steroid hormone, cortisol is derived from cholesterol via a steroidogenic process, which begins with the mobilization of free cholesterol and transport from intracellular stores into mitochondria where cholesterol will be metabolized to pregnenolone by the first enzyme of the pathway, the cytochrome P450 side chain cleavage enzyme complex (P450scct) (Papadopoulos, 1993; Stocco and Clark, 1986). Hormones, such as adrenocorticotropic and its second messenger cAMP, acting through the cAMP-dependent protein kinase (PKA), accelerate this process. Although cholesterol transport into mitochondria is the rate-determining step in steroid biosynthesis, steroid formation is also limited by the amount of the substrate cholesterol available. Cholesterol availability depends on cholesterol uptake by the LDL receptor and on the rate of its synthesis, involving the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase located in the cytoplasm, responsible for the conversion of HMG-CoA to mevalonate, a substrate of cholesterol.

Procaine (Fig. 1) is a widely used local anesthetic agent (Ruetsch et al., 2001) that has also been historically used to treat a variety of clinical conditions such as arthritis, depression, gastrointestinal distress, cardiac arrhythmia, hypertension, chronic skeletal pain, and allergies (Roka and LaJtha, 1950; Ellis, 1952; Traut, 1952). Procaine has been shown to elicit a variety of biological effects, including the inhibition of the hormone-stimulated cortisol production by bovine adrenocortical cells in vitro (Hadjian et al., 1982; Naguchi et al., 1990). In the present study we report that procaine and a procaine-based formulation (Anticort) decreased the dibutyryl cyclic AMP (dbcAMP)-induced steroid synthesis in adrenal cells and the circulating glucocorticoid levels in rats by reducing the HMG-CoA reductase mRNA expression and activity. This effect of procaine, although absent in testicular Leydig cells, was also seen in mouse liver cells.

**Materials and Methods**

**Materials.** Y1 mouse adrenal tumor cells were obtained from the American Type Culture Collection (Manassas, VA) and the MA-10 mouse Leydig tumor cells were generously given by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Mouse Hepa1-6 cells were obtained from the American Type Culture Collection. UT-1 cells were generously provided by Dr. J. L. Goldstein (Southwestern University, Dallas, TX). Fetal bovine lipoprotein-deficient serum was from INTRACEL Corp. (Frederick, MD). F-12K (Kain’s modification of Ham’s F-12) and DMEM culture media were purchased from the American Type Culture Collection and DMEM/Ham’s F-12K medium containing 15% horse serum and 2.5% FBS under 5% CO2 (Papadopoulos, 1993; Stocco and Clark, 1986). Y1 mouse adrenal tumor cells were cultured in DMEM/F-12 with 1% horse serum, and fetal bovine serum (FBS) were purchased from ICN Diagnostics (Flanders, NJ), and anti-glyceraldehyde-3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD). 3H-20α-hydroxyprogesterone, 3H-progesterone, 3H-cortisosterone, and 3H-mevalonolactone were purchased from PerkinElmer Life Sciences (Boston, MA) and 14C-HMG-CoA was obtained from Amersham Biosciences Inc. (Bucksinghamshire, UK). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay kit was purchased from Trevigen, the PepTag assay for nonradioactive detection of the PKA kit was purchased from Promega (Madison, WI), and the Varian Bond-Elut NH2 columns were obtained from Chrom Tech, Inc. (Apple Valley, MN). The pGL3 luciferase reporter vector, pRL-TK Luciferase vector, and dual-luciferase reporter assay system were from Promega. Trans-l-LT1 was from Mirus Corp. (Madison, WI), and restriction endonucleases were from New England Biolabs (Beverly, MA). Procaine hydrochloride and compactin were obtained from Sigma-Aldrich (St. Louis, MO). Anticort and placebo were obtained from Samaritan Pharmaceuticals, Inc. (Las Vegas, NV). Anticort is a formulation containing procaine hydrochloride, zinc sulfate heptahydrate (used to decrease the rate of absorption of procaine), ascorbic acid (used as an antioxidant), potassium benzoate (used as a preservative), and disodium phosphate. The placebo is the same formulation devoid of procaine hydrochloride. RNA STAT-60 was from Tel-Test, Inc. (Friendswood, TX). TaqMan Reverse Transcription Reagents, random hexamers, and SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA). All other chemicals used were of analytical grade and were obtained from various commercial sources.

**Animal treatment.** Male 80-day-old Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Rats were housed at the Georgetown University Research Resources Facility under controlled light and temperature, with free access to rat chow and water. They were housed in groups of three and acclimated to their new conditions for 2 days before treatment. All experimental protocols were reviewed and approved by the Georgetown University animal care and use committee. The procaine-based formulation Anticort (25–100 mg/kg) and placebo (both prepared by the University of Iowa School of Pharmacy, Iowa City, IA), were administered by gavage in 1-mL volume every day for a total of 8 days. Rats were sacrificed 24 h later. Corticosterone was measured in organic extracts (ethyl acetate/ether, 1:1, v/v) of the collected sera by radioimmunoassay (Amri et al., 1996) under conditions suggested by the supplier of the antisera, ICN Diagnostics Division (Orangeburg, NY).

**Cell Culture.** Y1 mouse adrenal tumor cells were cultured in F-12K medium containing 1.5% horse serum and 2.5% FBS under 5% CO2 (Brown et al., 1992). MA-10 mouse Leydig tumor cells were cultured in DMEM/F-12 medium supplemented with 5% FBS and 2.5% horse serum under 4% CO2 (Brown et al., 1992). Human adrenal tumor H295R cells were maintained in DMEM/F-12 medium supplemented with 1% ITS+ (insulin (1 µg/ml), transferrin (1 µg/ml), selenium (1 µg/ml), linoleic acid (1 µg/ml), and bovine serum albumin (1.25 mg/ml)), 2.5% Nuserum, and 1% penicillin-streptomycin at 37°C, 6% CO2 (Amri et al., 1996). Hepa-1 mouse hepatoma cells were cultured in DMEM supplemented with 10% FBS and UT-1 cells were cultured in DMEM/F-12 medium supplemented with 8% fetal bovine lipoprotein-deficient serum and 2% FBS plus 40 µM Compactin (Chin et al., 1982).

**Determination of Steroid Synthesis.** Y1 or MA-10 cells were cultured in 96-well plates (2 × 104 cells/well) for 18 h, and then treated with increasing concentrations of either procaine hydrochloride (0.1, 1, 10, and 100 µM) or Anticort (0.1, 1, 10, and 100 µM based on procaine content) for 48 h. Culture media were then changed and cells were stimulated with 1 mM dbcAMP for 24 to 48 h. The synthesis of 20α-OH progesterone and progesterone in Y1 and MA-10 cell media, respectively, were measured by radioimmunoassay (Brown et al., 1992). H295R human adrenal tumor cells were seeded in 48-well plates at 105 cells/well and incubated for 24 h. After removal of culture media, cells were incubated in the presence of procaine (0.1, 1, 10, and 100 µM) or Anticort (0.1, 1, 10, and 100 µM).
based on procaine content) for another 48-h period. At the end of the incubation period cells were treated with or without 1 mM dbcAMP for 48 h. Cortisol levels in the media were determined by radioligand assay previously described (Amri et al., 1996).

### Analysis of Mitochondrial Integrity/Cell Viability

Cell viability at the end of the incubation protocol described above was assessed using the mitochondrial integrity MTT assay (Tryvigen). Briefly, 10 μl of the MTT solution were added to the cells in 100 μl medium. After an incubation period of 4 h, 100 μl of detergent were added and cells were incubated overnight at 37°C. Formazan blue formation was quantified at 600 and 690 nm using the Victor quantitative detection spectrophotometer (EGG-Wallac, Gaithersburg, MD) and the results expressed as (OD600–OD690).

### PKA Activity Measurement

Y1 cells were cultured in 6-well plates (2 × 10⁵ cells/well) and treated as described above for steroid biosynthesis. At the end of the incubation period cells were washed twice with PBS and proteins extracted using an extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotonin). After centrifugation at 18,500g for 15 min, the supernatants were kept for PKA activity assay. Samples were processed using the PepTag assay for nonradioactive detection of PKA activity following the manufacturer’s recommendations (Promega).

### Immunoblotting

At the end of the treatment protocol described above, Y1 cells were washed two times with PBS, sonicated 15 s in extraction buffer, and centrifuged at 18,500g for 1 min at 4°C. Pellets were resuspended in ice-cold lysis buffer (1% Nonidet P-40 in extraction buffer), sonicated briefly, and incubated on ice for 1 h. After centrifugation (22,500g for 30 min, 4°C), the supernatant was mixed in sample buffer 6X (0.27 M SDS, 0.6 M dithiothreitol, 0.18 M bromphenol blue in 7 ml of 0.5 M Tris-HCl, pH 6.8, and 3 ml glycerol) and boiled for 5 min. Proteins were subjected to SDS-PAGE (4–20% gradient SDS-polyacrylamide gel) and electrohoretically transferred onto nitrocellulose membranes. The transblot sheets were blocked with 5% nonfat dry milk in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 overnight at 4°C. Membranes were then incubated with appropriately diluted primary antibodies, 1:800 for anti-P-GS luciferase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and enhanced chemiluminescence (Amersham Life, Arlington Heights, IL). The densities of the appropriate bands were determined using the OptiQuant Acquisition and Analysis software (PerkinElmer Life Sciences).

### HMG-CoA Reductase Assay

Y1 cells in 12-well plates (1 × 10⁵ cells/well) were treated with increasing concentrations of procaine hydrochloride (1, 10, and 100 μM) for 48 h. Cells were washed twice with ice-cold PBS and incubated with ice-cold assay buffer (0.1 M sucrose, 40 mM KH₂PO₄, 30 mM EDTA, 50 mM KCl, 5 mM dithiothreitol, 0.25% (v/v) Brij 96, at pH 7.4) on ice for 20 min. After centrifugation for 3 min at 14,000g (the supernatant were collected and used for HMG-CoA reductase activity assay. The total 150 μl assay mixture contained 100 to 200 μg/ml of protein and the NADPH-generating system (2.5 mM NADP, 20 mM glucose 6-phosphate, and 20 U/ml glucose 6-phosphate dehydrogenase). The reaction was started by adding substrate (14C-HMG-CoA in C₆H₂NO₄) and stopped after 45 min by adding 10 μl of 6 M HCl. 1H-meravonolactone was also added to the samples as an extraction recovery marker. After an additional 30-min incubation time, to allow complete lactonization of the product, the mixture was centrifuged. The supernatant was applied to a Bond-Elut NH₄ column and eluted with 1 ml of toluene/acetone (3:1). The eluate was discarded and an additional 4 ml of toluene/acetone was applied to the column and collected in a scintillation vial for counting 14C and 3H signals (Berkhout et al., 1990).

In separate experiments cells were disrupted by sonication and then treated with procaine. The direct effect of the treatment on HMG-CoA reductase activity in the homogenates was determined as described above.

### Real-Time Quantitative PCR (Q-PCR)

Cells cultured in 6-well plates for 18 h were treated with or without 10 or 100 μM hydrocortisone for 24 h. After treatment, cells were stimulated with 1 mM dbcAMP for 24 h. At the end of the incubation, total cell RNA was extracted using RNA STAT-60 (Tel-Test, Inc.) according to the manufacturer’s instructions. HMG-CoA reductase mRNA was quantified by Q-PCR using the ABI Prism 7700 sequence detection system (PerkinElmer/Applied Biosystems, Foster City, CA). The reverse transcription reaction was performed using TaqMan Reverse Transcription Reagents with 1 μg total RNA and random hexamers as primers for each reaction according to the manufacturer’s instructions. For quantifying mouse HMG-CoA reductase mRNA with Q-PCR, the primers were designed according to GenBank Accession Number BC 019782 using PE/AB Primer Express software, which is specifically designed for the selection of primers and probes. The forward primer was 5’-CCAGGTGTTGAGGAGGTGGTT-3’ (22 nucleotides), and the reverse primer was 5’-CGTCAACCATGGTCGGTAGT-3’ (23 nucleotides), respectively. The primers were synthesized by Bio-Synthesis (Lewisville, TX). Reactions were performed in a reaction mixture consisting of a 20-μl solution containing 10 μl SYBR Green PCR Master Mix and 1 μl primers mix (5 μM each), with 2 μl cDNA. The cycling conditions were 15 s at 95°C and 1 min at 60°C for 40 cycles following an initial step of 2 min at 50°C and 10 min at 95°C. AmpliTag Gold polymerase was activated at 95°C for 10 min. The 18S RNA was amplified at the same time and used as an internal control. To exclude the contamination of unspurious PCR products such as primer dimers, a melting curve analysis was applied to all final PCR products after the cycling protocol. Also, the PCR reactions without the reverse transcription reaction were performed for each sample to exclude genomic DNA contamination. The PCR products were collected and run on a 3% (w/v) agarose/TAE gel to confirm the product size. The threshold cycle (Ct) values for 18S RNA and samples were calculated using the PE/AB computer software. Ct was determined at the most exponential phase of the reaction. Relative transcript levels were calculated as x = 2 ΔΔCt, in which ΔΔCt = ΔCt – ΔC, and ΔCt = Ct(experiment) – Ct(controls). ΔC = Ct(control) – Ct(18s).

### Cloning of the Mouse HMG-CoA Reductase Promoter into pG3 Luciferase Reporter Vector and Transient Transfections

To examine the effects of procaine on HMG-CoA reductase gene transcription, the 383-nucleotide fragment (from –308 to +75) of HMG-CoA mouse reductase promoter was generated by PCR and subcloned into the pGL3 firefly luciferase (Photinus pyralis) reporter basic vector. DNA prepared from mouse embryo stem cells was used as template. The forward primer was 5’-ctctcggcttggtggggactc- cccgctgatg-3’ with the MluI site (in italics), and the reverse primer was 5’-ccgatctgggtggagactcctactgattgctag-3’ with the BgIII site (in italics). Amplified fragments were purified and ligated to the pGL3-basic vector between the MluI and BgIII sites and sequenced to verify the predicted sequence. The pRL-TK Renilla luciferase vector was used as a positive control to evaluate and normalize transfection efficiency.

Y1 cells were grown in 12-well plates at 2 × 10⁵ cells/well for 24 h. For transfection we used the Transk-1 reagent, following the manufacturer’s instructions. Plasmids at a ratio of pGL3 and pRL-TK vectors of 50:1 (mol/mol) were added into culture medium. After a 24-h incubation the cells were washed with 10 μM dexamethasone and then stimulated with 1 mM dbcAMP for an additional 24 h. After treatment, cells were harvested and promoter activity was determined using the Dual-Luciferase reporter assay system using a luminometer (Victor2; LKB-Wallac, Gaithersburg, MD).

### Measurement of HMG-CoA Reductase mRNA Stability

To examine the effects of procaine on HMG-CoA reductase mRNA stability, Y1 cells were treated with 1 or 10 μM procaine for 24 h followed by 24-h treatment with 1 mM dbcAMP. The decay of HMG-CoA reductase mRNA was subsequently determined by incubating the cells with and without actinomycin D (10 μg/ml) for an additional
2- or 4-h time period. Longer exposures to actinomycin D were toxic to the cells. Total cellular RNA was isolated and HMG-CoA reductase mRNA levels were measured by Q-PCR as described above.

**Protein measurement.** Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as a standard.

**Statistics.** Statistical analysis was performed by one-way analysis of variance (ANOVA) and unpaired Student’s *t* test using the INSTAT 3.00 package from GraphPad (San Diego, CA).

## Results

**Procaine Inhibits the dbcAMP-Induced Steroid Formation in Mouse and Human Adrenal Cell Lines.** Treatment of Y1 cells with dbcAMP increased 20α-hydroxyprogesterone production by approximately 4-fold (Fig. 2A; *p* < 0.001). Procaine and the procaine-based formulation Anticort decreased in a dose-dependent manner the dbcAMP-induced 20α-hydroxyprogesterone production (Fig. 2A), with IC$_{50}$ values close to 0.1 μM. The compounds tested did not affect basal steroid formation by Y1 cells (data not shown). Moreover, none of the compounds used affected cell viability as determined using the MTT assay (Fig. 2B).

In H295R cells, dbcAMP increased cortisol synthesis by 3-fold (Fig. 3A, *p* < 0.001). Procaine and Anticort inhibited the dbcAMP-stimulated cortisol production in a dose-dependent manner (*p* < 0.01 by ANOVA) as shown in Fig. 3A, without affecting basal cortisol production (not shown). Surprisingly, cells exposed to dbcAMP showed a dramatic decrease in cell viability, as determined by the MTT assay. However, cell numbers were not decreased following dbcAMP treatment, suggesting that in this case changes in MTT may reflect mitochondrial function rather than cell viability. Interestingly, both procaine and Anticort (Fig. 3B) protected against the dbcAMP-induced change of mitochondrial function. Because mitochondrial function is critical for steroid formation (Stocco and Clark, 1996), the data presented in Fig. 3A were corrected by the number of cells with intact mitochondrial function (MTT activity shown in Fig. 3B). Figure 3C shows that both procaine and Anticort exert a strong inhibitory effect on the dbcAMP-stimulated cortisol production, with IC$_{50}$ values lower than 10 μM.

In contrast to adrenal cells, procaine did not affect the dbcAMP-induced progesterone synthesis in MA-10 mouse Leydig tumor cells (Fig. 4A). The treatment did not affect MA-10 cell viability either (Fig. 4B).

**Anticort Reduces Circulating Corticosterone Levels in Male Sprague-Dawley Rats.** Eight-day treatment of adult male rats with the procaine-based formulation Anticort reduced serum corticosterone levels by approximately 50% in a significant manner (*p* < 0.05), as assessed by ANOVA (Fig. 5). Similar results were obtained with adult mice treated with procaine hydrochloride (data not shown).

**Effect of Procaine on various Steps of the Steroidogenic Pathway.** Considering the effect of procaine on the dbcAMP-stimulated steroid formation, the effect of this compound on PKA activity was investigated. PKA activity was measured using a nonradioactive detection kit based on the PKA-specific substrate, PepTag A1 peptide (L-R-R-A-S-L-G). Figure 6 shows that 1 μM procaine, which inhibited by 90% the dbcAMP-stimulated steroid formation (Fig. 2A), has no significant effect on the dbcAMP-stimulated PKA activity.

The hydrosoluble cholesterol substrate of the P450scx, 22R-hydroxycholesterol, induced a 7.5-fold increase in 20α-hydroxyprogesterone formation (Fig. 7A). As shown in Fig. 2A, 1 μM procaine reduced the dbcAMP-induced steroid formation by 90%. However, procaine did not inhibit the effect of 22R-hydroxycholesterol on steroidogenesis (Fig. 7A). In addition, procaine did not modify the expression of the P450scx enzyme as assessed by immunoblot analysis of cell extracts (Fig. 7B).

The data presented above (Figs. 6 and 7) indicated that the effect of procaine is beyond the activation of PKA and before cholesterol metabolism to final steroid products. We examined the effect of procaine on two proteins involved in the transport of cholesterol into mitochondria, the peripheral-type benzodiazepine receptor (PBR) and the steroidogenesis acute regulatory protein (STAR), using the same 48-h treatment protocol with procaine. These experiments showed that 1 μM procaine did not affect either the ligand-binding characteristics of PBR (R$_{max}$ = 27 ± 3 pmol/mg protein and K$_D$ = 1.8 nM in control cells versus R$_{max}$ = 29 ± 4 pmol/mg protein and K$_D$ = 1.7 nM in procaine-treated cells) or the levels of the mature 30-kDa STAR protein (Fig. 7C), which was induced by 2.5-fold following a 3-h dbcAMP treatment. In light of these results, we investigated whether cholesterol synthesis itself was affected by procaine.

![Fig. 2. Effect of procaine and procaine-based formulation Anticort on the dbcAMP-induced 20α-hydroxyprogesterone synthesis (A) and cell viability (B) in Y1 mouse adrenal tumor cells. Cells were incubated with increasing concentrations of the indicated compounds for 48 h. At the end of the incubation media were changed and cells were incubated with 1 mM dbcAMP for 48 h. The treatment with the procaine-based formulation Anticort was based on procaine content. A, 20α-Hydroxyprogesterone levels in cell media were determined by radioimmunoassay. The effect of the compounds on steroid formation was statistically significant (*p* < 0.001 by ANOVA). B, cell viability was determined using the MTT assay as described under Materials and Methods. Results shown are means ± S.D. from three independent experiments performed in triplicate.](https://doi.org/10.1016/S0022-2836(98)96043-2)
Procaine Inhibits the HMG-CoA Reductase Activity and mRNA Expression. The cholesterol substrate mevalonate was used to assess the effect of procaine on cholesterol synthesis. Figure 8A shows that 1 μM procaine did not inhibit the dbcAMP- and mevalonate-supported 20α-hydroxyprogesterone formation, indicating that procaine may act at the level of mevalonate synthesis by the HMG-CoA reductase enzyme. HMG-CoA reductase activity was determined in Y1 cells. Procaine reduced in a dose-dependent manner HMG-CoA reductase activity in these cells (Fig. 8B). The percentages of inhibition for the concentration of 1, 10, and 100 μM procaine were 44%, 72%, and 70%, respectively, and the effect of the treatment was highly significant (p < 0.001 by ANOVA). To assess whether the effect of procaine is due to a direct effect on the enzyme activity, Y1 cells were sonicated and treated with procaine. No direct effect of procaine on HMG-CoA reductase activity was observed (10.1 ± 0.9 pmol/min/mg protein control versus 9.9 ± 0.01, 10.3 ± 0.6, and 10.1 ± 0.1 pmol/min/mg protein in the presence of 1, 10, and 100 μM procaine, respectively).

Based on these data we examined the effect of procaine on HMG-CoA reductase mRNA expression levels measured by Q-PCR and using 18S RNA as internal standard. Treatment of Y1 cells with dbcAMP for 24 h induced by 1.8-fold (p < 0.001) the HMG-CoA reductase mRNA expression (Fig. 9A). Pretreatment of the cells for 24 h with procaine reduced in a dose-dependent manner HMG-CoA reductase mRNA levels (p < 0.01 by ANOVA), bringing them close to the basal levels (Fig. 9A). Detailed time course studies indicated that a 6-h treatment with procaine was the earliest time point when the compound inhibited the dbcAMP-induced HMG-CoA reductase mRNA expression, and that this effect was enhanced when cells were pretreated for 24 h with procaine (data not shown). Although a trend of inhibition of HMG-CoA reductase mRNA expression was seen in UT-1 cells, a Chinese hamster ovary cell clone containing high levels of HMG-CoA reductase, selected to grow in the presence of compactin, an HMG-CoA reductase inhibitor (Chin et al., 1982), this effect was not significant (Fig. 9B). However, procaine inhibited the dbcAMP-induced HMG-CoA reductase mRNA levels in Hepa1-6 mouse liver hepatoma cells (Fig. 9C) in a significant manner (p < 0.01 by ANOVA). Figure 9, B and C also show that in both the UT-1 and Hepa1-6 cells, treatment with dbcAMP induced the HMG-CoA reductase mRNA levels in a significant manner (p < 0.001 and p < 0.05, respectively).

Procaine Does Not Affect HMG-CoA Reductase mRNA Transcription, but Accelerates Its Decay. The results presented above suggest that procaine exerts a direct effect on HMG-CoA reductase mRNA stability or DNA transcription. Thus, we decided to extend these studies by examining the effects of procaine treatment on HMG-CoA reductase gene transcription. For that we isolated the 383-nucleotide mouse HMG-CoA reductase proximal promoter fragment. The eventual role of the promoter elements present within this region implicated in the down-regulation of the HMG-CoA reductase gene transcription by procaine was examined with transient transfection experiments. Y1 cells transfected with the HMG-CoA reductase(-308/+75)-pGL3 construct were treated with and without 10 μM procaine for 24 h and then stimulated with dbcAMP for an additional 24 h. The results obtained indicated that procaine induced a consistent minor, but not significant, inhibition of HMG-CoA reductase gene transcription (Fig. 10A).

RNA stabilization can account in part for changes in HMG-CoA reductase mRNA levels. We tested whether procaine treatment affected HMG-CoA reductase mRNA stability by exposing the cells to 1 and 10 μM procaine for 24 h followed by 24-h treatment with 1 mM dbcAMP, and then measuring the decay of HMG-CoA reductase mRNA by incubating the
cells with actinomycin D for up to 4 h. Results shown in Fig. 10B suggest that both concentrations of procaine tested accelerate the HMG-CoA reductase mRNA decay.

**Discussion**

Y1 mouse adrenal tumor cells have been extensively used to understand the mechanisms underlying adrenal steroid formation. In these cells, 20α-hydroxyprogesterone, resulting from the conversion of progesterone by 20α-hydroxylase, is the major steroid formed (Brown et al., 1992). In this study, we showed that procaine inhibits the cAMP-induced 20α-hydroxyprogesterone increase in Y1 cells. However, procaine did not affect basal 20α-hydroxyprogesterone production by the cells. Moreover, procaine inhibited the cAMP-induced steroid synthesis in a dose-dependent manner. This modulatory effect of procaine on the cAMP-induced steroid formation was not restricted to mouse Y1 adrenal cells, and it was also observed in the H295R human adrenal tumor cells, which synthesize cortisol as the main steroid product. Human H295R tumor cells, however, were less sensitive to procaine than mouse Y1 cells. These results confirm and extend previous observations reporting that procaine lowered the steroidogenic effect of cholinergic muscarinic stimulation (Hadjian et al., 1982) and dbcAMP (Noguchi et al., 1990) on bovine adrenocortical cells. These data, together with the finding that procaine did not affect basal steroid formation, suggested that procaine exerts its modulatory activity only in the presence of a stimulus. Interestingly, procaine did not affect the cAMP-induced steroid formation in a mouse Leydig cell line, indicating the tissue specificity of the response. In support of this finding, the tissue-specific transcriptional regulation of key protein components of the steroidogenic pathway has been demonstrated (Simpson et al., 1991). Nevertheless, this modulatory effect of procaine on adrenal cells has to be assessed in brain and ovarian cells.
The effect of procaine on both mouse and human adrenal cells was replicated using a procaine-based formulation, Anticort, which, on a procaine molar basis, inhibited the cAMP-induced steroidogenesis to the same extent as procaine hydrochloride alone. None of the compounds tested affected adrenal cell viability, determined using the MTT assay. In contrast, in human adrenal tumor cells, the treatment with dbcAMP induced a decrease in MTT levels, indicating either an effect on cell viability or an effect of the nucleotide analog on mitochondrial diaphorase activity. Considering that we did not observe an effect on cell numbers, it is likely that the effect seen is due to a direct effect on mitochondrial function. This effect was not seen with Y1 cells and it was reversed by treatment with either procaine or Anticort.

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The effect of procaine and Anticort was not restricted to in vitro models. Treatment of rats and mice for 8 days with Anticort decreased serum corticosteroid levels by 50%. Thus, there is enough corticosterone remaining to support the glucocorticoid-dependent functions. This is also supported by the fact that procaine has been used safely in the clinic for more than 40 years. Moreover, we should also consider the possibility that at least 50% of the measured corticosteroid levels may reflect the normal “unstressed” condition. As the rats were not preconditioned, the handling-induced stress was probably responsible for the stimulation of the corticosterone synthesis (Kant et al., 1989). Indeed, surveying the literature for circulating corticosterone levels in rats, we found that there is a large variation in the reported values ranging from 4 to 40 ng/ml. Thus, in vivo treatment with Anticort does not affect the basal adrenal function, but rather it controls the stress-induced glucocorticoid levels, thus maintaining lower “normal” circulating corticosterone levels. It should be noted, however, that procaine has been also described to decrease the release of corticotropin-releasing factor previously induced in a model of cerebral hemorrhage in rats (Plotsky and Vale, 1984) and to decrease the release of adrenocorticotropic in a model of surgically induced stress in the dog (Ganong et al., 1976). Such a central effect of procaine on the hypothalamus and pituitary cannot be excluded to explain the decrease of the corticosterone concentrations we observed in the in vivo experiments, in addition to a direct effect on the adrenal cells, reinforcing the interest of procaine and its derivatives as corticosteroid modulating agents. In contrast to these findings, Kling et al. (1994) reported that...
Procaine induced the hypothalamic corticotropin-releasing hormone release, leading to the activation of the pituitary-adrenal axis. The rapid metabolism of procaine in the blood by esterases, the distinct routes of administration used, and the differences in the model systems used (stressed versus nonstressed) may account for the discrepancies between these in vivo studies. In the studies presented herein, we administered procaine by gavage in a preparation formulated to improve its stability and bioavailability.

In search of the mechanism mediating the action of procaine on cAMP-induced adrenal steroidogenesis, we first looked for an effect on the cAMP-induced PKA activity, mediating the effect of hormones on cholesterol transport into mitochondria and steroidogenic enzyme expression. Quantification of the dbcAMP-stimulated Y1 cell PKA activity was measured as described under Materials and Methods. The results shown are means ± S.E.M. (n = 6) from two independent experiments. B, Y1 cells were treated with or without 1 and 10 μM procaine for 24 h followed by 24-h treatment with 1 mM dbcAMP. At the end of the incubation cells were washed and exposed to actinomycin D for 2 or 4 h. Cells were scraped and PBR mRNA levels were measured by Q-PCR as described under Materials and Methods. Results shown are the means ± S.E.M. of two separate experiments, each performed in triplicate (n = 6), expressed as the ratio of the cAMP-induced versus control HMG-CoA reductase mRNA levels.

Fig. 9. Effect of increasing concentrations of procaine on HMG-CoA reductase mRNA levels in Y1, UT-1, and Hepa1-6 cells. Cells were treated with increasing concentrations of procaine for 24 h followed by treatment with 1 mM dbcAMP for 24 h. At the end of the incubation, total cell RNA was extracted and HMG-CoA reductase mRNA was quantified by Q-PCR as described under Materials and Methods. Results are shown as means ± S.E.M. from three independent experiments. The effect of procaine on the dbcAMP-induced HMG-CoA reductase mRNA levels was statistically significant in Y1 and Hepa1-6 cells (p < 0.001 and p < 0.05, respectively, by ANOVA) and not significant in UT-1 cells.

Procaine Regulates HMG-CoA Reductase mRNA
that P450osc enzyme levels were not affected by procaine. Taken together, these data suggest that procaine and its derivatives might affect the amount of cholesterol available for steriodogenesis. Such an effect may be due either to a change in the rate of cholesterol transfer from intracellular stores into mitochondria or to an effect on cholesterol synthesis. Procaine had no effect on the expression levels of PBR and StAR, the two key regulatory proteins mediating the transfer of cholesterol into mitochondria (Papadopoulos, 1993; Stocco and Clark, 1996). The finding that addition of the substrate of cholesterol synthesis mevalonate in the medium resulted in abolishing the inhibitory effect of procaine on the dbcAMP-stimulated steroid formation suggested that procaine's site of action is at a step before mevalonate synthesis.

The rate-limiting enzyme in mevalonate and cholesterol biosynthesis is HMG-CoA reductase. Treatment of the cells with increasing concentrations of procaine resulted in the dose-dependent decrease of HMG-CoA reductase activity, assessed by the transformation of $^{14}$C-HMG-CoA into $^{14}$C-mevalonate. Maximal inhibition was achieved in the presence of 10 µM procaine. The IC$_{50}$ value of the effect of procaine was 1 µM, a concentration higher than that required to inhibit by 50% corticosteroid formation, suggesting that in addition of its effect on HMG-CoA reductase activity procaine might exert additional effects in adrenal cells through not yet identified mechanisms. Considering the absence of a direct effect of procaine on HMG-CoA reductase activity measured in adrenal cell extracts, we hypothesized that procaine may act on HMG-CoA reductase mRNA levels. Indeed, treatment of Y1 cells with dbcAMP resulted in increased HMG-CoA mRNA levels, in agreement with previous findings that hormones regulate HMG-CoA reductase enzyme gene expression (Ness and Chambers, 2000). Procaine inhibited in a dose-dependent manner the dbcAMP-induced HMG-CoA reductase mRNA expression levels without affecting basal HMG-CoA mRNA levels. This finding is in agreement with the effect of procaine on the cAMP-induced steroid formation. To examine the tissue specificity of the effect of procaine on HMG-CoA mRNA expression we used two cell types, the UT-1 and Hepa1-6 cells. The UT-1 cell line is a clone of Chinese hamster ovary cells (CHO-K1) selected to grow in the presence of compactin, a competitive inhibitor of HMG-CoA reductase. These cells have a 500-fold higher level of HMG-CoA reductase activity and protein than normal cells (Chin et al., 1982). Hepa1-6 cells are mouse liver hepatoma clones used herein because liver is the main organ in cholesterol synthesis. Treatment of both UT-1 and Hepa1-6 cells with dbcAMP induced HMG-CoA mRNA expression. Treatment of the cells with procaine resulted in the dose-dependent decrease of HMG-CoA mRNA levels. This effect was minor and not significant in the UT-1 cells but robust in the Hepa1-6 cells, suggesting that there is a tissue specificity of the effect of procaine on HMG-CoA reductase mRNA expression and activity.

In vitro transfection studies of the proximal HMG-CoA reductase promoter into Y1 cells followed by procaine treatment did not significantly affect the cAMP-induced HMG-CoA reductase mRNA levels, suggesting that procaine did not directly affect the HMG-CoA reductase gene expression. However, we cannot exclude the possibility that procaine may have an effect on HMG-CoA reductase gene expression, either acting at a distant element of its promoter or that it is not obvious, because it overlaps with the effect of other positively regulated elements present in the proximal promoter. Further studies on HMG-CoA reductase mRNA stability indicated that procaine might act by accelerating the HMG-CoA reductase mRNA decay.

The finding that procaine regulates HMG-CoA reductase mRNA levels is a novel observation, and the data indicating that liver cholesterol formation might be regulated by procaine are intriguing findings. At present, statins are used as the major therapeutic means for hypercholesterolemia (Stein, 2003) because they occupy a portion of the binding site of HMG-CoA, thus blocking access of this substrate to the active site of HMG-CoA reductase (Istvan and Deisenhofer, 2001). In addition, statins are in clinical trials for their use to slow AD progression, a disease where hypercholesterolemia seems to play a critical role (Waldman and Kitharides, 2003). Procaine’s mechanism of action via the reduction of the cAMP-induced HMG-CoA mRNA levels offers an alternative approach to statins for regulating the HMG-CoA reductase activity. It is of interest that local anesthetics, including procaine, were previously shown to affect steroid biosynthesis at a step beyond mevalonate formation (Bell and Hubert, 1980), most likely by inhibiting the cholesterol esterase (Traynor and Kunze, 1975) and cholesterol acyltransferase (Bell, 1981) enzyme activities. Our data do not exclude such actions of procaine or other effects that this molecule might exert at a postmevalonate step, effects that might be tissue-specific, as those described on adrenal and liver HMG-CoA reductase enzymes.

Elevated concentrations of cortisol have been reported to be associated with many diseases and to worsen the prognosis. In contrast to the detrimental effects of high levels of cortisol in the pathologies described above, maintenance of the basal cortisol levels is necessary for the maintenance of basic biological functions. Glucocorticoids regulate the metabolism of proteins, carbohydrates, and lipids, and are essential to the adaptation to acute physical stressors (Munck and Naray-Fejes-Toth, 1994). Development of compounds that block the excessive glucocorticoid synthesis without affecting the basal steroid formation has proven to be a difficult task, because it requires the identification of a modulator of an activity rather than an inhibitor. We presented herein evidence that procaine lowered the hormone-stimulated corticosteroid formation by adrenal cells. Procaine was found to act by reducing the levels of the rate-limiting enzyme HMG-CoA reductase mRNA, leading to reduced activity and decreased cholesterol and corticosteroid biosynthesis. Procaine did not affect basal corticosteroid formation, suggesting that only pathological states of high glucocorticoid formation would be affected. Because procaine hydrochloride is the ester of diethylaminoethanol and para-aminobenzoic acid, and as such it can be easily hydrolyzed in the body, future development of stable procaine derivatives exhibiting similar properties and no cell toxicity might be good drug candidates. We propose that such “corticosteroid synthesis modulators” may be valuable for the treatment of high-cortisol diseases such as AIDS, multiple sclerosis, AD, depression, and Cushing’s hypertension either alone or in combination with disease-specific therapies.
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