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ACTH induces TIMP-1 expression and inhibits collagenase in adrenal cortex cells☆

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Abstract

To identify genes that are induced by corticotropin (ACTH) in adrenal cortex cells, we carried out a differential hybridization screening of adrenal cortex cDNA libraries. Some of the clones we identified represented tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA. We examined ACTH dependence of the expression of TIMP-1 in vitro in cultured bovine adrenocortical cells, and in ACTH-treated rats. Northern blot analysis of total RNA from cells showed that the level of TIMP-1 mRNA increases sharply within 3 h after ACTH stimulation. Since TIMP-1 inhibits some cell matrix metalloproteinases (MMPs) of the collagenase type, we examined the effect of ACTH on collagenase activity in bovine adrenocortical cells. Exposure of confluent cultures to ACTH for 24 h showed dose-dependent inhibition of collagenase activity. Northern blot analysis of total RNA from rat adrenal zona fasciculata-reticularis and zona glomerulosa showed that in both of these zones TIMP-1 expression was induced within 12 h after ACTH injection. Long-term (9 days) treatment with ACTH increased TIMP-1 mRNA levels nearly sixfold in zona fasciculata-reticularis. Overall, our results show that ACTH causes induction of TIMP-1 and suppression of collagenase activity, and suggest that ACTH may modulate the activities of MMPs and hence cell matrix remodeling.

Keywords: ACTH; Collagenase regulation; TIMP-1 mRNA; Bovine adrenocortical cells; Rat adrenal cortex

1. Introduction

Biosynthesis and secretion of steroid hormones from steroidogenic cells are dependent on the stimulation of these cells by their respective trophic hormones. Effects of trophic hormones are mediated via second messengers and changes in the expression of specific enzymes and transcription factors (Hanukoglu, 1992; Waterman and Bischof, 1996; Cooke, 1999; Hu et al., 2001). The list of trophic hormone-induced genes includes genes that encode diverse steroidogenic enzymes, regulatory proteins and mitochondrial genes that are involved in energy production (Viard et al., 1992; Raikhinstein and Hanukoglu, 1993; Lehoux et al., 1998; Wood and Strauss, 2002). To identify genes that are induced by ACTH in adrenal cortex cells, we carried out a differential hybridization screening of adrenal cortex cDNA libraries. Some of the clones we identified represented tissue inhibitor of metalloproteinase 1 mRNA.

TIMP-1 is a member of the TIMP family that includes four proteins, TIMP-1, through TIMP-4. All four proteins share conserved structural features, bind to and inhibit cell matrix metalloproteinases (MMPs), but differ in their regulation and specificities to inhibit MMPs (Brew et al., 2000). MMP family includes about 25 members, many of which function as collagenases (Sternlicht and Werb, 2001; Lauer-Fields et al., 2002). MMPs are involved in the turnover of extracellular matrix structural proteins, and appear to play roles in cell matrix remodeling, cell growth and apoptosis during both normal developmental and pathological processes of tissue restructuring such as wound healing. TIMPs are the major regulators of the activities of MMPs and the balance between MMPs and TIMPs is considered to be a major factor determining the turnover of extracellular matrix proteins (Gomez et al., 1997; Smith et al., 2002). TIMP-1 over-expression is associated with attenuated tumor growth, reduced metastasis and suppression of angiogenesis (Bloomston et al., 2002; Ikenaka et al., 2003).

☆ The sequence of bovine tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA has been submitted to GenBank under accession number AY295346.

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In our pilot studies on identification of ACTH-induced mRNAs in adrenocortical cells, six clones represented TIMP-1 mRNA. In a previous single study on the expression of TIMPs in adrenocortical cells, 10 nM ACTH was found to induce TIMP-2 expression but no effect was observed on TIMP-1 expression (Quirin et al., 1999). Hence, we decided to examine the effect of ACTH on expression of TIMP-1 and on collagenase activity in vitro cultured cells, and in vivo ACTH-treated rats. Results of our in vitro studies showed that after addition of ACTH, TIMP-1 expression is induced within hours. In addition, ACTH was also observed to repress collagenase activity. In vivo studies showed that ACTH induces TIMP-1 expression also in the adrenal cortex of rats. Overall, our results suggest that ACTH may modulate the activities of MMPs and hence cell matrix remodeling.

2. Materials and methods

2.1. Cell culture, reagents and animals

All chemicals used were reagent grade. For in vitro studies we used ACTH 1-24 (Organon). For in vivo studies in rats we used Synacthen Depot ACTH generously provided by Ciba-Geigy, Montreal, Canada.

Primary cultures of bovine adrenal cortex cells were prepared and grown to confluence in Dulbecco's Modified Eagle's Medium and Ham's F12 (1:1) containing 12.5% horse serum and 2.5% fetal calf serum, 100 μg/ml bovine serum albumin, 100 μM ascorbic acid, 1 μM α-tocopherol, 200 nM sodium selenite, 20 nM insulin (Hanukoglu et al., 1990). Confluent cultures were stimulated with ACTH as described previously (Raikhinstein and Hanukoglu, 1993). Control cultures were grown similarly in medium without ACTH.

Two-month-old Long Evans male rats were purchased from Charles River (St. Constant, Quebec, Canada). They were fed Purina rat chow and tap water ad libitum. The separation of the rat adrenal zona glomerulosa from the zona fasciculata-reticularis containing the medulla was done as described by Giroud et al. (1956).

2.2. RNA isolation and Northern blot analysis

Total RNA from bovine tissues was isolated as previously described (Hanukoglu et al., 1990). Total RNA from cultured cells was isolated by Gough's method (Gough, 1988). For Northern blot analyses, total RNA was separated on 1.2% agarose-formaldehyde gel, electro-transferred to GeneScreen nylon membrane and reacted with [^{32}P]-labeled cDNA probes as described (Raikhinstein and Hanukoglu, 1993). The Northern blots were also reacted with a cDNA probe for mitochondrial 16S rRNA taken as a control. This probe was observed as the best control to ascertain that there are equal quantities of RNA on each lane of Northern blots of RNA from various tissues (Raikhinstein and Hanukoglu, 1993).

Northern blot autoradiograms were quantitated using a densitometer, or scanned and quantitated using Scion Image version of NIH Image. Each blot was repeated with RNA isolated from at least two independent experiments with essentially identical results.

2.3. Cloning, sequencing and cDNA probes

Bovine adrenal cortex cDNA library was screened, and cDNA inserts isolated and sequenced as previously described (Raikhinstein and Hanukoglu, 1993). The cDNA probes were [^{32}P]-labeled using a random primer method.

2.4. Collagenase assay

To assay collagenase activity, bovine adrenocortical cells were cultured and grown to confluence as described above in 60 mm plates. Collagenase activity in harvested cells was determined as described previously (Reich et al., 1995). Results are expressed as the measured intensity relative to that of control.

3. Results

3.1. Isolation and characterization of cloned TIMP-1 cDNAs

The cDNA probes for cDNA library screening were synthesized from total RNA isolated in three experiments from bovine adrenocortical cell cultures incubated with fresh medium for 6 h with or without ACTH. Sequences of six ACTH-inducible clones (picked out of about 100,000 recombinant plaques) were identical with bovine ovarian TIMP-1 mRNA (Freudenstein et al., 1990). The sequence of our longest clone (clone no. 518, GenBank accession no. AY295346) at the 5′ end was 89 nucleotides longer than the previously reported sequence for bovine ovarian TIMP-1 cDNA (Freudenstein et al., 1990).

3.2. TIMP-1 mRNA expression in bovine tissues and adrenocortical cells

To determine the tissue specificity of expression of TIMP-1, we reacted TIMP-1 cDNA probe with a Northern blot of total RNA from 11 bovine tissues. The results showed ubiquitous expression of TIMP-1 in all tissues (Fig. 1). Yet, the highest level expression was observed in the corpus luteum, followed by the adrenal.

In the absence of ACTH stimulation, the level of expression of TIMP-1 mRNA in primary cultures of adrenocortical cells was much higher than adrenal cortex tissue (Fig. 2). The probing of the same blot with control cDNA probes

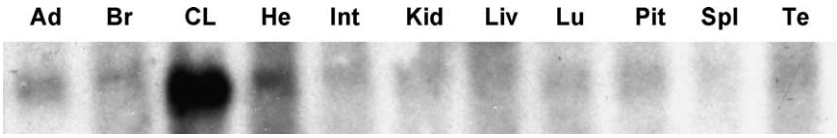

Fig. 1. TIMP-1 mRNA expression in bovine tissues as detected by Northern blot analysis with [^{32}P]-labeled TIMP-1 cDNA. Each lane of the gel included 20 μg total RNA from the following tissues adrenal cortex (Ad), brain (Br), corpus luteum (CL), heart (He), intestine (Int), kidney (Kid), liver (Liv), lung (Lu), pituitary (Pit), spleen (Spl) and testis (Te).

for cytochrome *c* oxidase subunit I mRNA, and mitochondrial 12S rRNA showed similar levels in both cultured cells and tissues (Fig. 2). As expected, the levels of P450C17 mRNA were markedly lower in cultured cells versus adrenal cortex tissue, as the expression of this gene is ACTH dependent.

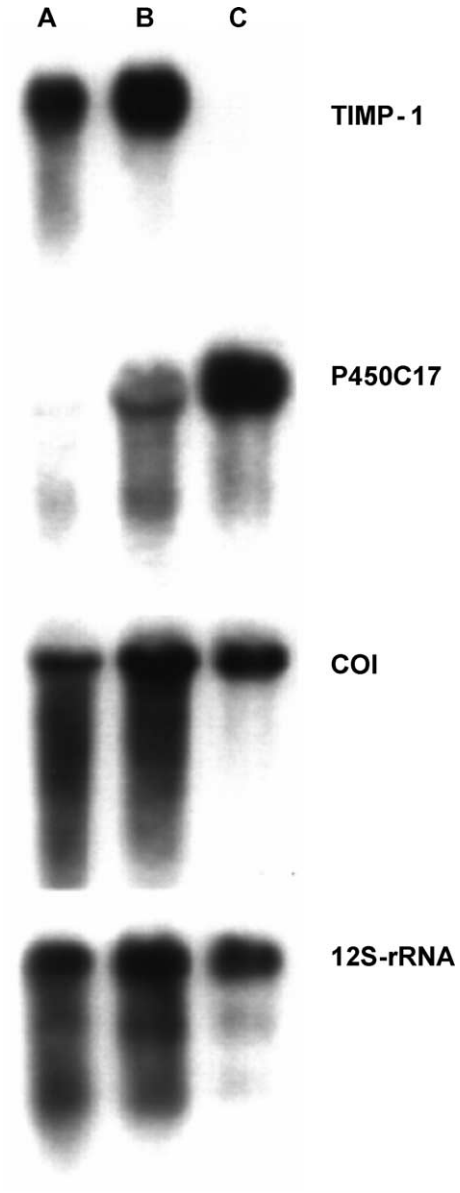

Fig. 2. Levels of expression of selected mRNAs in bovine adrenocortical cells in primary culture versus bovine adrenal cortex tissue. Lanes A and B: two independent preparations of RNA from cultured cells. Lane C: RNA from adrenal cortex tissue. All lanes included 20 μg total RNA. The Northern blot analysis was performed with the following probes: TIMP-1, P450c17, cytochrome oxidase subunit I, and 12S mitochondrial rRNA.

To determine the time course of ACTH induction of TIMP-1 mRNA, we carried out Northern blot analysis of total RNA from ACTH stimulated cells in primary culture. The results showed that the level of TIMP-1 mRNA increases sharply to a peak within 3 h after ACTH stimulation, then gradually drops to control levels and below over 48 h (Fig. 3).

3.3. Effect of ACTH on collagenase activity in adrenocortical cells

Since TIMP-1 inhibits some MMPs of collagenase type, we examined the effect of ACTH on collagenase activity in bovine adrenocortical cells. Twenty-four hour exposure of confluent cultures to increasing concentrations of ACTH showed a clear dose-dependent inhibition of collagenase activity in the range of 10–1000 nM ACTH (Fig. 4) ($P < 0.05$ at 30 nM, and $P < 0.01$ at higher concentrations of ACTH). Cells maintained in the absence of ACTH showed

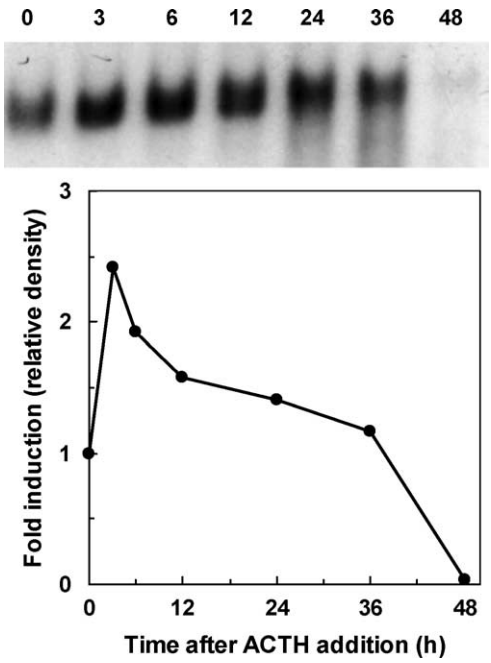

Fig. 3. Time course of ACTH effect on TIMP-1 mRNA in bovine adrenocortical cells. Primary cultures were incubated with ACTH for the number of hours shown above each lane. The amount of total RNA was 20 μg in all lanes. Autoradiogram of the RNA blot was quantitated by Scion Image. The densities are as expressed relative to 0 h density taken as 1.

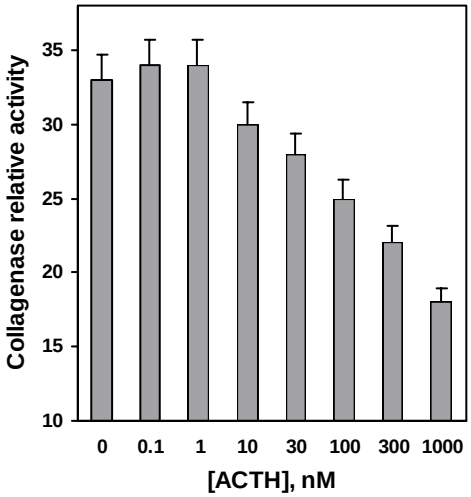

Fig. 4. Effect of ACTH concentration on collagenase activity in bovine adrenocortical cells. Primary cultures of adrenocortical cells were grown to confluence. At time zero, the medium was replaced with fresh medium either without ACTH (0), or with increasing concentrations of ACTH (from 0.1 to 1000 nM). After 24 h the cells were harvested and frozen in liquid nitrogen for later collagenase activity assay. Each point represents the average of results of three independent plates.

increasing amounts of collagenase activity, suggesting continuous secretion of collagenase (Fig. 5). Yet, the addition of ACTH to the medium resulted in suppression of collagenase activity over the entire 48 h period examined (Fig. 5).

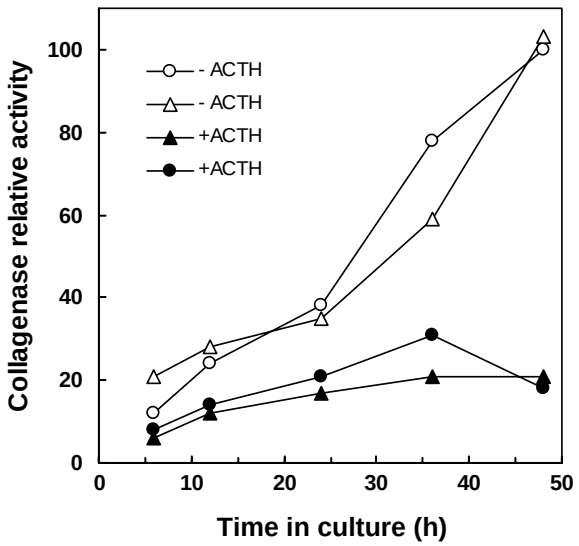

Fig. 5. Time course of ACTH effect on collagenase activity in bovine adrenocortical cells. Primary cultures of adrenocortical cells were grown to confluence. At time zero, the medium was replaced with fresh medium either without ACTH (−ACTH), or with 1 μM ACTH (+ACTH). The cells were harvested after 6, 12, 24, 36 or 48 h and frozen in liquid nitrogen for later collagenase activity assay. The graph shows the results of two independent experiments. Each point is the average of results of three independent plates.

3.4. Effect of in vivo ACTH treatment on TIMP-1 expression in rat adrenals

To determine whether the inductive effect of ACTH on TIMP-1 expression is observed also in vivo, we carried out a series of studies in rats. Northern blot analysis of total RNA from rat adrenal zona fasciculata-reticularis and zona glomerulosa showed that in both of these zones TIMP-1 expression was induced within 12 h after ACTH injection (Fig. 6). Densitometric analysis of autoradiograms indicated an average of twofold induction of TIMP-1 mRNA level, 12 h after ACTH injection.

To examine the long-term effect of ACTH groups of rats were injected ACTH twice a day for 9 days. In these studies, TIMP-1 mRNA levels increased significantly (5.6-fold by densitometric analysis) in zona fasciculata-reticularis (Fig. 7). In zona glomerulosa samples, the intensity of the hybridization signal was too weak for reliable quantitation.

Daily injection of glucocorticoid analog, dexamethasone, led to a significant increase in TIMP-1 mRNA level (threefold by densitometric analysis) in zona fasciculata-reticularis.

4. Discussion

In this study, we identified and isolated cDNA clones for TIMP-1 mRNA induced in response to ACTH stimulation in bovine adrenocortical cells. Using the cloned cDNA as a probe we studied the expression of TIMP-1 mRNA in both in vitro and in vivo conditions. To examine the physiological significance of TIMP-1 induction, we also studied ACTH effect on collagenase activity since a major function of TIMP-1 is inhibition of MMPs.

Our results indicate that in both bovine and rat adrenal cortex cells, ACTH induces the expression of TIMP-1 mRNA within hours, with a time course that parallels the induction of mitochondrial and microsomal steroidogenic P450 system enzymes (Hanukoglu et al., 1990; Raikhinstein and Hanukoglu, 1994). Cultured bovine adrenocortical cells represent mainly zona fasciculata of adrenal. Studies on finely separated zones of rat adrenals indicate that ACTH induces TIMP-1 in zonae fasciculata-reticularis and glomerulosa, but long-term sustained effect is observed only in the zona fasciculata-reticularis of the cortex (Fig. 7).

To examine the effect of blocking endogenous secretion of ACTH, we studied a group of rats that received glucocorticoid analog dexamethasone at a dose known to block ACTH secretion. The results revealed that dexamethasone by itself stimulated TIMP-1 expression, though at a level lower than that observed with ACTH alone (Fig. 7). This effect of dexamethasone was observed in zona fasciculata-reticularis but not in zona glomerulosa. This finding raises the possibility that ACTH effects on TIMP-1 expression and collagenase activity may be partly secondary to the hormone's effect on steroidogenesis. Overall, the in vivo findings show

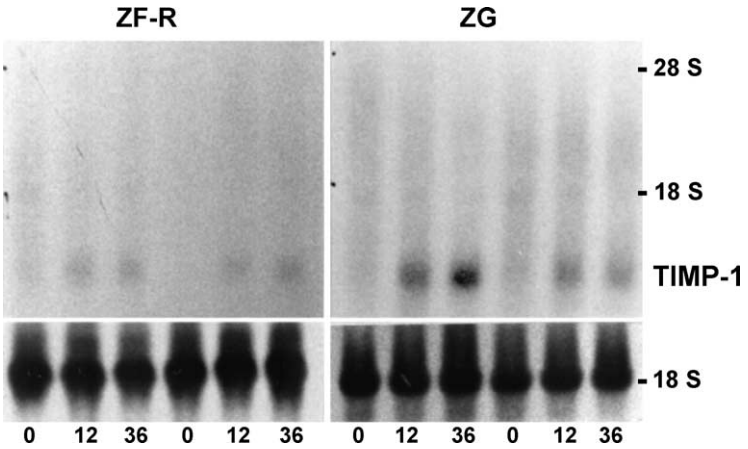

Fig. 6. Effect of ACTH on TIMP-1 mRNA levels in rat adrenal zones. Groups of four rats were injected subcutaneously with ACTH (3 units/100 g body weight) at 0, 12 and 24 h. Controls (0 h) were injected vehicle only. Total adrenal tissue RNA was isolated 0, 12 or 36 h after the first injection. Amount of total RNA was 15 μg in all lanes. Left panel: RNA from zona fasciculata-reticularis (ZF-R). Right panel: RNA from zona glomerulosa (ZG). Northern blots were hybridized with labeled cDNA probe for TIMP-1 mRNA (upper panels). After stripping the blots were reacted with cDNA probe for 18S rRNA (bottom panels).

evidence of significant endocrine regulation of TIMP-1 in zona fasciculata-reticularis.

Among 11 bovine tissues we examined, highest levels of TIMP-1 mRNA were detected in corpus luteum that undergoes rapid tissue remodeling. Immunofluorescent studies in rat ovary show that TIMP-1 is mainly present in the blood vessels, thecal cells, luteal capillaries and the luteal cell plasma membrane with minor staining in the granulosa cells (Bagavandoss, 1998). In both animals and humans, TIMP-1 plays an essential role in ovulation and fertility (Tsafriri and Reich, 1999; Nothnick, 2001).

ACTH, while enhancing TIMP-1 expression, repressed collagenase activity in a concentration-dependent manner (Figs. 4 and 5). These concomitant effects on TIMP-1 induction and collagenase repression show a consistent picture of strong ACTH inhibition of MMP activity in adrenal cortex cells. The rapid induction of TIMP-1 within hours after ACTH, suggests that TIMP-1 is involved in acute regulation of adrenal MMPs. Enhanced levels of TIMP-1 mRNA in primary cultures versus intact adrenal tissue (Fig. 2) show that TIMP-1 expression is drastically affected by cell environment, consistent with its role in extracellular matrix remodeling.

The predominant actions of ACTH are directed to stimulate the synthesis and secretion of steroid hormones. The results presented in this study reveal a novel aspect of ACTH

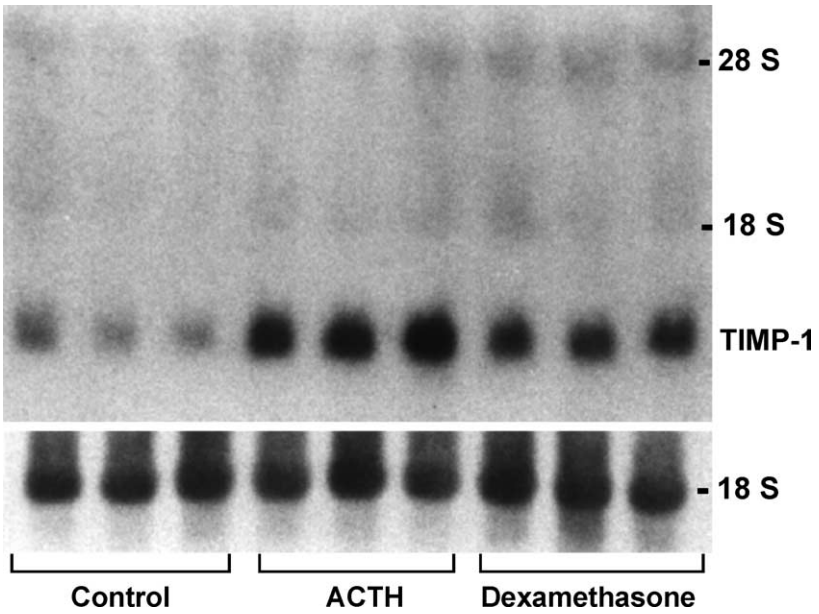

Fig. 7. Effect of long-term (9 days) ACTH and dexamethasone treatment on TIMP-1 mRNA levels in rat adrenal zona fasciculata-reticularis (ZF-R). ACTH (3 units/100 g body weight) was injected twice a day for 9 days. Dexamethasone (0.2 mg/100 g body weight) was administered daily for 5 days. Total tissue RNA was isolated 12 h after the last injection. Analyses were performed as indicated in the legend of Fig. 6.

action that should result in inhibition of tissue remodeling. These results are also relevant to understanding the biochemical consequences of Cushing syndrome of excess ACTH secretion that should result in strong repression of tissue remodeling in adrenal cells and may be other peripheral target cells of ACTH.

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