

Steroidogenic acute regulatory protein gene expression, steroid-hormone secretion and proliferative activity of adrenocortical cells in the presence of proteasome inhibitors: *In vivo* studies on the regenerating rat adrenal cortex

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Received November 27, 2007; Accepted January 14, 2008

Abstract. Previous studies have shown that proteasome inhibitors promote the accumulation of steroidogenic acute regulatory protein (StAR) in cultured rat adrenocortical cells. Unexpectedly, this response was associated with a moderate lowering in the corticosterone secretion and proliferation rate of cultured cells. Hence, we studied the effects of proteasome inhibitors MG115 and MG132 on the secretion and proliferative activity of the regenerating adrenal cortex in rats 5 days after surgery. Animals were given two subcutaneous injections of 0.15 or 1.5 nmol/100 g of inhibitors 24 and 12 h before decapitation. Real-time PCR and Western blotting showed that StAR expression, both mRNA and protein, was markedly lower in regenerating adrenals than in the intact gland of sham-operated rats. Neither MG115 nor MG132 affected StAR expression in regenerating gland. Inhibitors induced a slight decrease in the plasma concentrations of aldosterone and corticosterone, but did not significantly alter metaphase index of the regenerating adrenal cortex. Our findings provide the first evidence that down-regulation of StAR occurs during the early stages of adrenal regeneration. Moreover, this suggests that the steroidogenic pathway is more sensitive to proteasome inhibitors than that regulating proliferative activity of regenerating adrenal cortex in the rat.

Introduction

Steroidogenic acute regulatory protein (StAR) plays a crucial role in steroidogenesis, because it regulates the translocation of cholesterol from the outer to the inner mitochondrial membrane (1,2), the rate-limiting step of this process. In steroidogenic cells, the 37 kDa StAR preprotein is rapidly cleaved to the mature 30 kDa protein, and the half-lives of StAR preprotein trapped in the cytosol and mitochondria have been estimated at 15 min and 4.8 h, respectively (3-6). Due to its short half-life, StAR preprotein must be produced continuously if steroidogenesis is to be maintained.

In most cultured mammalian cells under optimal nutritional conditions, ~80-90% of the protein breakdown occurs via the proteasome pathway (7). Compelling evidence indicates that the bulk of short-lived regulatory proteins, including StAR, is degraded by the ubiquitin-proteasome system (5,8). Moreover, experimental data suggested stabilization of regulatory proteins upon treatment with proteasome inhibitors (7). In keeping with this contention, findings showed that the treatment with proteasome inhibitors promotes StAR protein accumulation and StAR mRNA expression in cultured rat preovulatory granulosa and adrenocortical cells, respectively (8,9). However, these changes were coupled to a rise in progesterone output from granulosa cells, but to a lowering in corticosterone production from adrenocortical cells.

Of interest, a 24-h exposure to proteasome inhibitors was found to decrease the proliferation rate of cultured adrenocortical cells (9). In this connection, we remind that beacon, a ubiquitin-like protein (10-12) expressed in the rat adrenal cortex (13,14), has been found to inhibit the growth of cultured adrenocortical cells (15). Furthermore, it has been recently demonstrated that in the regenerating rat adrenal cortex the expression of the beacon gene is inversely correlated with the proliferative activity of adrenocortical cells (16). It, therefore, seemed worthwhile to study the effects of two proteasome inhibitors on the secretion and proliferative activity of the regenerating rat adrenal cortex.

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Key words: regenerating adrenal cortex, proteasome inhibitors, steroidogenic acute regulatory protein expression, steroid-hormone secretion, proliferation activity, rat

Materials and methods

Animals and experimental design. Adult female rats (130–140 g body weight), bred in our laboratory facilities, were kept under a 14:10 h light-dark cycle (illumination onset at 6.00 a.m.) at 23°C, and maintained on a standard diet and tap water *ad libitum*. Under ether anesthesia, the left adrenal gland of 48 rats was enucleated, and the right adrenal was removed (17). A group of animals was sham-operated. Rats were given 0.9% NaCl to drink for 3 days, and were decapitated 5 days after surgery. Rats that underwent adrenal enucleation were divided into 6 equal groups (n=8), four of which were given two subcutaneous (s.c.) injections of 0.15 or 1.5 nmol/100 g of the proteasome inhibitors MG115 or MG132 (7) dissolved in 0.2 ml of 0.3% dimethyl sulfoxide (DMSO) 24 and 12 h before decapitation. The other two groups served as a control, and received two sc injections of 0.9% NaCl or DMSO. Three hours before sacrifice animals were given an intra-peritoneal injection of 0.1 mg/100 g of vincristine. MG115, MG132 and DMSO were purchased from Sigma-Aldrich Corp. (St. Louis, MO), and vincristine was provided by Gedeon-Richter (Budapest, Hungary). Animals were decapitated at 11:00 a.m., and trunk blood was collected in the presence of EDTA (1 mg/ml). Plasma was separated and stored at -36°C. Regenerating adrenals of three rats in each group were fixed in Bouin's solution, and the others were frozen and stored at -80°C. The experimental protocol was approved by the local Ethics Committee for Animal Studies, and experiments were carried out according to the Italian Law on the protection of laboratory animals.

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was extracted from the frozen regenerating adrenals and intact gland of sham-operated animals, and reverse transcribed to cDNA (18–21). Semiquantitative real-time PCR was performed as previously detailed (22–24) in a Roche LightCycler 2.0 with software version 4.0, using the following primers: i) StAR sense (684–703), 5'-CCTGAGCAAAGCGG TGTCAT-3' and antisense (850–870), 5'-GCAAGTGGCTGG CGAACTCTA-3' (amplifon, 187 bp; accession number, NM-03155); and ii) HPRT (hypoxanthine guanine phosphoribosyl transferase 1) sense (391–412), 5'-CAGTCAACGGGGG ACATAAAAG-3' and antisense (515–536), 5'-ATTTTGG GGCTGTACTGCTTGA-3' (amplicon, 146 bp; accession number, NM-01258). The PCR program was: denaturation step at 95°C for 10 min, 45 cycles of three amplification steps (95°C for 10 sec, annealing at 58°C for 5 sec, and extension at 72°C for 10 sec), and melting curve at 60–90°C with a heating rate of 0.1°C/sec. All samples were amplified in duplicate, and HPRT was used as the housekeeping gene to normalize data, its mRNA expression being taken equal to one.

Western blotting. Proteins were extracted from frozen specimens (16), and Western blotting was carried out as previously detailed (25), using the following primary antibodies: i) rabbit anti-rat StAR polyclonal antibody (Calbiochem), 1:10,000 dilution overnight at room temperature; and ii) mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) monoclonal antibody (Abcam),

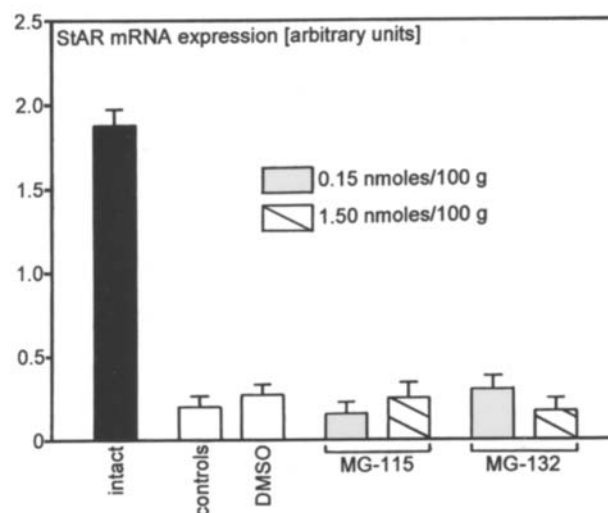


Figure 1. Semiquantitative real-time PCR assay of StAR mRNA expression in the regenerating adrenal cortex of control and proteasome inhibitor-treated rats. Bars are means \pm SEM (n=5). $P < 0.01$ from intact adrenals of sham-operated rats.

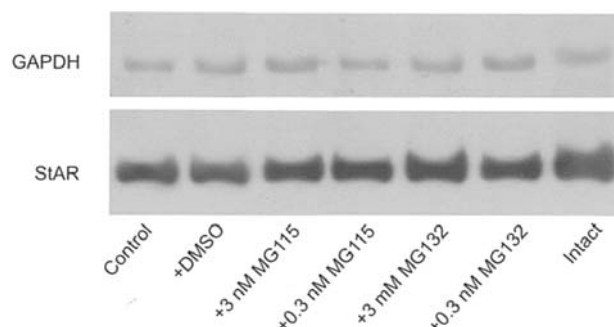


Figure 2. Western blot of mature 30 kDa StAR protein in exemplary regenerating adrenal cortices of control and proteasome inhibitor-treated rats, and in intact rat adrenals of a sham-operated rat. GAPDH protein bands of ~37 kDa are shown in the upper panel.

1:2,000 dilution overnight at room temperature. Secondary peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG polyclonal antibodies (1:25,000 and 1:10,000 dilution, respectively; 60 min at room temperature) were obtained from Amersham Biosciences. Immunoreactive bands were detected by ECL Advanced Western blotting detection kit (Amersham Life Science). Bands were densitometrically analyzed by TotalLab 2.0 Software, and pixel intensity of GAPDH bands were used to normalize the amount of StAR protein.

Metaphase index. Regenerating adrenals fixed in Bouin's solution were embedded in paraffin and sectioned at 5–6 μ m of thickness. Sections were stained with hematoxylin and eosin, and metaphase index (number of metaphases per 1,000 cells) was calculated at a magnification of $\times 400$, by counting 5,000 cells in each regenerating gland (16).

Hormone assay. Aldosterone and corticosterone were extracted from plasma, and measured by RIA, as detailed earlier (26,27).

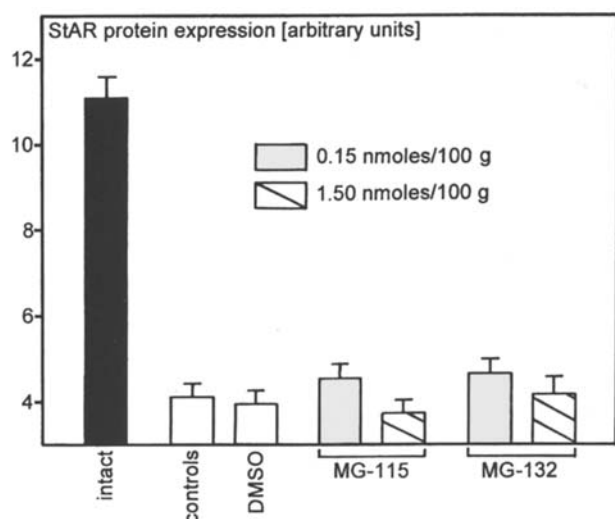


Figure 3. Semiquantitative Western blot assay of StAR protein expression in the regenerating adrenal cortex of control and proteasome inhibitor-treated rats. Bars are means \pm SEM (n=5). $P<0.001$ from intact adrenals of sham-operated rats.

Aldosterone RIA: sensitivity, 5 pg/ml; intra- and interassay CVs, 5 and 7%, respectively. Corticosterone RIA: sensitivity, 50 pg/ml; intra- and interassay CVs, 7 and 9%, respectively.

Statistics. Data were expressed as means \pm SEM, and the statistical significance of the differences among experimental groups was estimated using ANOVA, followed by the Duncan's multiple range test.

Results

Real-time PCR showed that StAR mRNA expression was notably lower in the regenerating adrenals than in the intact glands (Fig. 1). Western blotting demonstrated a single mature StAR-protein 30 kDa band in adrenal tissue (Fig. 2), and StAR protein expression was markedly lower in the regenerating than intact gland specimens (Fig. 3). Neither MG115 nor MG132 significantly affected StAR gene expression, as mRNA and protein, in the regenerating adrenals.

The higher dose of MG132 decreased the blood aldosterone concentration in rats with regenerating adrenals, and both doses of MG115 lowered the plasma level of corticosterone (Fig. 4). The proteasome inhibitors did not significantly alter the metaphase index of regenerating adrenocortical cells (Fig. 5).

Discussion

Adrenal regeneration after enucleation and contra-lateral adrenalectomy is a useful experimental model of rapid adrenocortical growth. Following enucleation with removal of inner cortex and medulla, remnant parenchymal cells adherent to the gland connective capsule proliferate and restore structural adrenocortical zonation within 4-6 weeks. The growth of adrenal cortex during the early stages of regeneration can be divided into two phases: an initial differentiation period (from

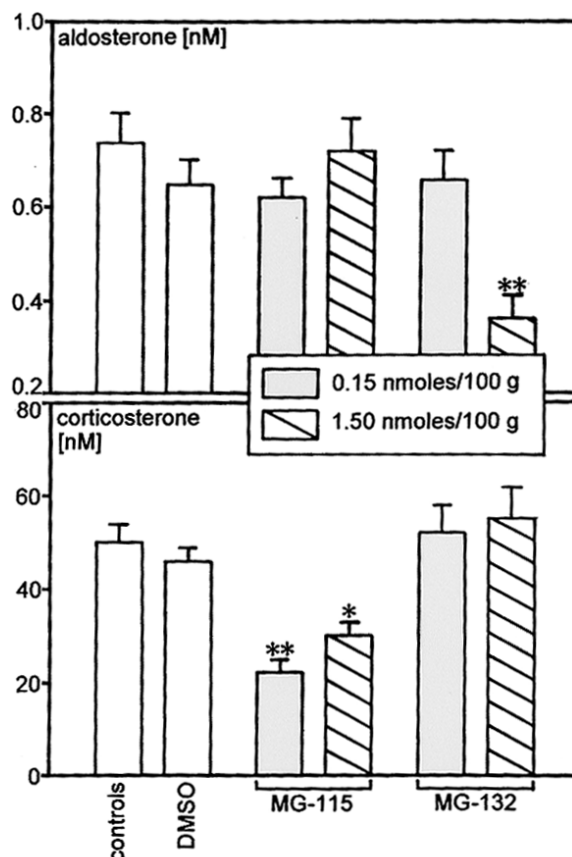


Figure 4. Effects of proteasome inhibitors on the plasma concentrations of aldosterone and corticosterone of rats hearing regenerating adrenal cortex at day 5 after surgery. Bars are means \pm SEM (n=8). * $P<0.05$ and $P<0.001$ from control group.

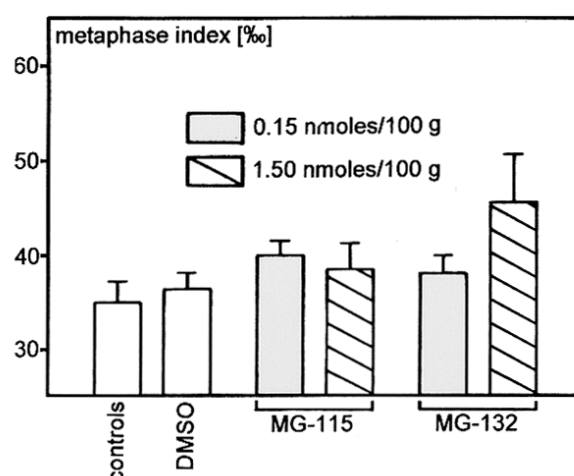


Figure 5. Lack of effect of proteasome inhibitors on the proliferative activity of regenerating rat adrenal cortex at day 5 after surgery. Bars are means \pm SEM (n=3).

days 0 to 3), and a subsequent high-proliferation period (from days 4 to 7), which are coupled to a marked decrease in the steroid-hormone secretion. From day 8 post-enucleation proliferative activity starts to decrease, and the secretory activity of the regenerating adrenal is slowly restored (28-34).

The striking down-regulation of the expression of the rate-limiting step enzyme of steroidogenesis StAR during the early stages of adrenal regeneration fits well with the reduced gland steroidogenic capacity. It is reasonable to conceive that at day 5 post-surgery highly proliferating adrenocortical cells are unable to produce adequate amounts of StAR, whose expression rapidly decrease due to its rather short half-life (3-6).

The administration of MG115 and MG132 did not affect either StAR protein content in or the proliferative activity of regenerating adrenocortical cells. This is a rather unexpected finding because proteasome inhibitors have been found to induce accumulation of StAR protein in cultured steroid secreting cells and to inhibit their proliferative activity (9). Moreover, MG115 has been reported to cause a complete blockade of G1/S and metaphase transitions, as well as a delayed progression through S phase in various cell types cultured *in vitro* (34). Several *in vitro* experiments showed that relatively high concentrations of proteasome inhibitors are needed to inhibit proteasome-dependent StAR protein degradation (5,6,8). Hence, our present negative findings may depend on the relatively low doses of inhibitors administered in our *in vivo* experiments, coupled to the rapid inactivation of the inhibitors due to their rather short half-life (7).

However, under the experimental conditions applied MG132 and MG115 administration decreased plasma concentrations of aldosterone and corticosterone, respectively, in rats at day 5 of adrenal regeneration. This observation appears to accord well with the previously described inhibitory effect of proteasome inhibitors on steroid secretion from freshly dispersed and cultured rat adrenocortical cells (9). In light of this, we suggest that the steroidogenic pathway is more sensitive to proteasome inhibitors than that regulating proliferative activity of regenerating adrenocortical cells in the rat.

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