

# Up-regulation of adrenomedullin receptor gene expression in activated local stem cells during rat adrenal regeneration

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**Abstract.** Previous studies showed that adrenomedullin (AM) gene expression was up-regulated in the regenerating rat adrenal cortex after enucleation and contra-lateral adrenalectomy, the effect being significant at day 1 after surgery and peaking between days 3 and 7. Using the same experimental model, we investigated by real time-polymerase chain reaction the mRNA expression of the AM receptor components: calcitonin receptor-like receptor (CRLR) and receptor activity-modifying proteins (RAMP)<sub>2</sub> and <sub>3</sub>. At time 0 (60 min after enucleation; control group), the CRLR mRNA content was ~2- and 5-fold higher than that of RAMP<sub>2</sub> and RAMP<sub>3</sub>, respectively. No significant changes in CRLR mRNA expression were observed in relation to the time elapsed from enucleation. RAMP<sub>2</sub> and RAMP<sub>3</sub> mRNAs did not exhibit significant changes at day 1 after surgery, but underwent a marked increase between days 3 and 7. The mRNA content of the two RAMPs decreased at days 14 and 28, although remaining significantly higher than that of the controls. These findings indicate that the AM receptor subtypes AM<sub>1</sub>-R (CRLR-RAMP<sub>2</sub>) and AM<sub>2</sub>-R (CRLR-RAMP<sub>3</sub>) are up-regulated in enucleated adrenals, and the hypothesis is advanced that this effect depends on the increased local production of AM. The concerted increase in AM and its receptor expression would greatly improve the autocrine-paracrine mechanism(s) by which AM favors proliferation of zona glomerulosa stem cells during adrenal regeneration.

## Introduction

Many lines of evidence suggest localization of adrenocortical stem cells in the zona glomerulosa (ZG), either adherent to the connective capsule or at the border with the zona

fasciculata, the so called zona intermedia (1,2). Under normal conditions, the renewal rate of adrenocortical cells is very low, so that it is conceivable that most stem cells are dormant. A useful experimental model of rapid adrenocortical growth, and consequently of strong activation of local stem cells, is rat adrenal regeneration after enucleation (3-5). Recent studies demonstrated that adrenomedullin (AM) gene expression is up-regulated in the adrenal parenchyma during the early stages of regeneration (6,7).

AM is a multi-functional regulatory peptide, that acts via a calcitonin receptor-like receptor (CRLR). CRLR selectivity for AM depends on its interaction with the members of a family of receptor activity-modifying proteins (RAMPs): RAMP<sub>1</sub> generates calcitonin gene-related peptide receptors, while RAMP<sub>2</sub> and RAMP<sub>3</sub> give rise to two AM receptors, referred to as AM<sub>1</sub>-R and AM<sub>2</sub>-R (8,9). AM and its receptors are expressed in the mammalian ZG, and AM has been shown to stimulate proliferation of ZG cells (10-13). Hence, it is conceivable that the increased production of AM in adrenal tissue after enucleation may favor the gland's regeneration.

Obviously, this effect of AM would be more intense if AM gene overexpression were coupled to that of its receptor genes, but earlier studies did not examine this possibility (6,7). It, therefore, seemed worthwhile to investigate by semiquantitative real time-reverse transcription (RT)-polymerase chain reaction (PCR) the expression of CRLR, RAMP<sub>2</sub> and RAMP<sub>3</sub> mRNAs at the early, middle and late stages of adrenal regeneration.

## Materials and methods

**Experimental design.** Adult male Sprague-Dawley rats (200-300 g body weight) were obtained from Charles-River (Como, Italy), and the experimental protocol was approved by the local Ethics Committee for Biomedical Studies. Under ether anesthesia, the left adrenal glands of 28 rats were enucleated and the contra-lateral glands were removed. Surgery was carried out by dorsal approach without opening the peritoneum (6). Enucleation consisted of an incision to the adrenal capsule, and extrusion and complete removal of adrenal parenchyma; only ZG cells adherent to the capsule (*bona fide* adrenocortical stem cells) remained *in situ*. Rats were given an intramuscular injection of amplital (20 mg/100 g) immediately after surgery and maintained on a standard diet and 0.9% NaCl saline to drink. They were decapitated at day 0 (60 min

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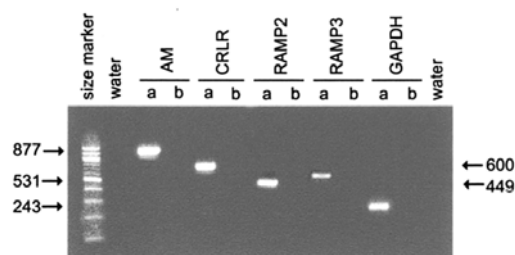


Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat AM, CRLR, RAMP2, RAMP3 and GAPDH-specific primers from RNA of an exemplary control rat regenerating adrenal cortex. Primer sequences were as follows: pAM sense-418-5', 5'-ACTCAGAGCA CAGCCAGAT-3' and pAM antisense-1249-3', 5'-AAGTTGTAGAGGAT GGGGTTG-3' (877 bp); CRLR sense-609-5', 5'-CTGGGACGGATGGCT ATG-3' and CRLR antisense-1208-3', 5'-CAGGCAGGAAGCAGAGGA-3' (600 bp); RAMP2 sense-104-5', 5'-GCTGTTACTGCTGCTGTTGC-3' and RAMP2 antisense-552-3', 5'-CATCGCCGCTTTACTCTC-3' (449 bp); RAMP3 sense-78-5', 5'-CTGCTTTGTGGTGAGTGTGC-3' and RAMP3 antisense-608-3', 5'-TCAGGAGGACTTTGACACGA-3' (531 bp); and GAPDH sense-83-5', 5'-GGGCTGCCTTCTTGTGAC-3' and GAPDH antisense-325-3', 5'-CGCCAGTAGACTCCACGACA-3' (243 bp). First lanes were loaded with 200 ng of a size marker (Marker VIII; Boehringer, Mannheim, Germany). No amplification of PCR mixture without prior RT of RNA is shown as negative control.

after enucleation, control group), 1, 3, 5, 7, 14 and 28 after surgery (4 animals for each time point), and their regenerating adrenals were promptly removed, frozen and stored at  $-80^{\circ}\text{C}$ .

**RT-PCR.** Total RNA was extracted from regenerating adrenal cortexes, and reverse transcribed to cDNA (14-16). Conventional PCR was carried out as previously described (17,18), using the following program: a predenaturation step at  $95^{\circ}\text{C}$  for 8 min to activate Taq DNA polymerase, followed by 35 cycles of denaturation at  $95^{\circ}\text{C}$  for 60 sec, annealing at  $60^{\circ}\text{C}$  for 45 sec and extension at  $72^{\circ}\text{C}$  for 90 sec. Primer sequences and the predicted sizes of amplicons are indicated in the legend of Fig. 1. To rule out the possibility of amplifying genomic DNA, one PCR was performed without prior RT of the RNA. Detection of the PCR amplification products was carried out by size fractionation on 2% agarose gel electrophoresis. The specificity of the PCR was verified by sequencing analysis (19).

**Real time-PCR.** Semiquantitative real time-PCR was carried out as previously detailed (20-23) in a Bio-Rad I-Cycler iQ Detection system (Bio-Rad Laboratories, Milan, Italy). Briefly, reactions were performed in 25  $\mu\text{l}$  of final volume solution containing 200 mM specific primers (Fig. 1 legend), 12.5  $\mu\text{l}$  iQ SYBR-Green Supermix (Bio-Rad Laboratories) and 2  $\mu\text{l}$  of RT-reaction solution. The PCR program consisted of a denaturation step at  $95^{\circ}\text{C}$  for 3 min, 35 cycles of two amplification steps ( $95^{\circ}\text{C}$  for 15 sec and  $60^{\circ}\text{C}$  for 30 sec) and melting curve at  $60-90^{\circ}\text{C}$  with a heating rate of  $0.5^{\circ}\text{C}/10$  sec. The specificity of amplification was tested at the end of each run by real time-melting analysis, using the I-Cycler iQ Software 3.0. All samples were amplified in duplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene; its mRNA expression equated to 1.

**Statistics.** The results were expressed as the means  $\pm$  SEM ( $n=4$ ), and the statistical significance of differences among experimental groups was estimated using ANOVA, followed by the Duncan's multiple range test.

## Results

Conventional RT-PCR detected the expression of AM, CRLR, RAMP2 and RAMP3 mRNAs in the control regenerating adrenal cortex (time 0) (Fig. 1). Real time-PCR showed that at time 0, the CRLR, RAMP2 and RAMP3 mRNA/GAPDH mRNA ratios were  $\sim 1$ , 0.5 and 0.2, respectively (Fig. 2). No significant changes were found in the CRLR mRNA expression in the regenerating adrenal parenchyma in relation to the time elapsed from enucleation (Fig. 2A). Conversely, both RAMP2 and RAMP3 mRNAs displayed marked increases between day 3 and day 7 after surgery ( $\sim 22$ - and 9-fold, respectively). The expression of RAMP2, but not RAMP3, was still above the control value at day 14, and at day 28 the mRNA of both RAMPs was not different from the controls (Fig. 2B and C).

## Discussion

Previous studies have shown that the activation of local stem cells during the early differentiative (from day 0 to day 3) and proliferative stages (from day 4 to day 7) of enucleation-induced adrenal regeneration is coupled to a marked increase in AM expression as mRNA and protein (7). The hypothesis has been advanced that relative hypoxia, ensuing from the disruption of the adrenal vascular bed, and the local increased release of inflammatory cytokines, caused by surgical adrenal manipulation, may be involved in this response. The marked increase in the blood concentration of ACTH due to the drop in glucocorticoid secretion following enucleation and contra-lateral adrenalectomy does not seem to play a major role, because up-regulation of AM gene expression also occurs in regenerating adrenals of rats where contra-lateral adrenal was spared and the level of circulating ACTH was in the normal range.

The possibility that hypoxia and inflammatory cytokines may induce up-regulation of AM receptor expression appears to be unlikely. In fact, the rise in RAMP2 and RAMP3 expression occurred only at day 3 from enucleation, while a marked increase in the AM gene transcription and translation was observed as early as 24 h post surgery (7). This sizeable temporal delay strongly suggests that up-regulation of RAMP2 and RAMP3, and consequently of AM<sub>1</sub>-R and AM<sub>2</sub>-R, depends on the increased local production of AM. According to this view, findings are available showing that under various pathological and experimental conditions a strict positive correlation exists between AM and RAMP2/3 expression in cardiomyocytes (24-27). Moreover, recent evidence showed that AM ( $10^{-8}$  M) enhanced AM<sub>1</sub>-R expression in cultured immortalized human microvascular endothelial cells (28).

However, there are many relevant differences between these last findings and our present ones, probably connected to the different tissues and experimental models employed. In the human endothelial cells cultured *in vitro* the response to

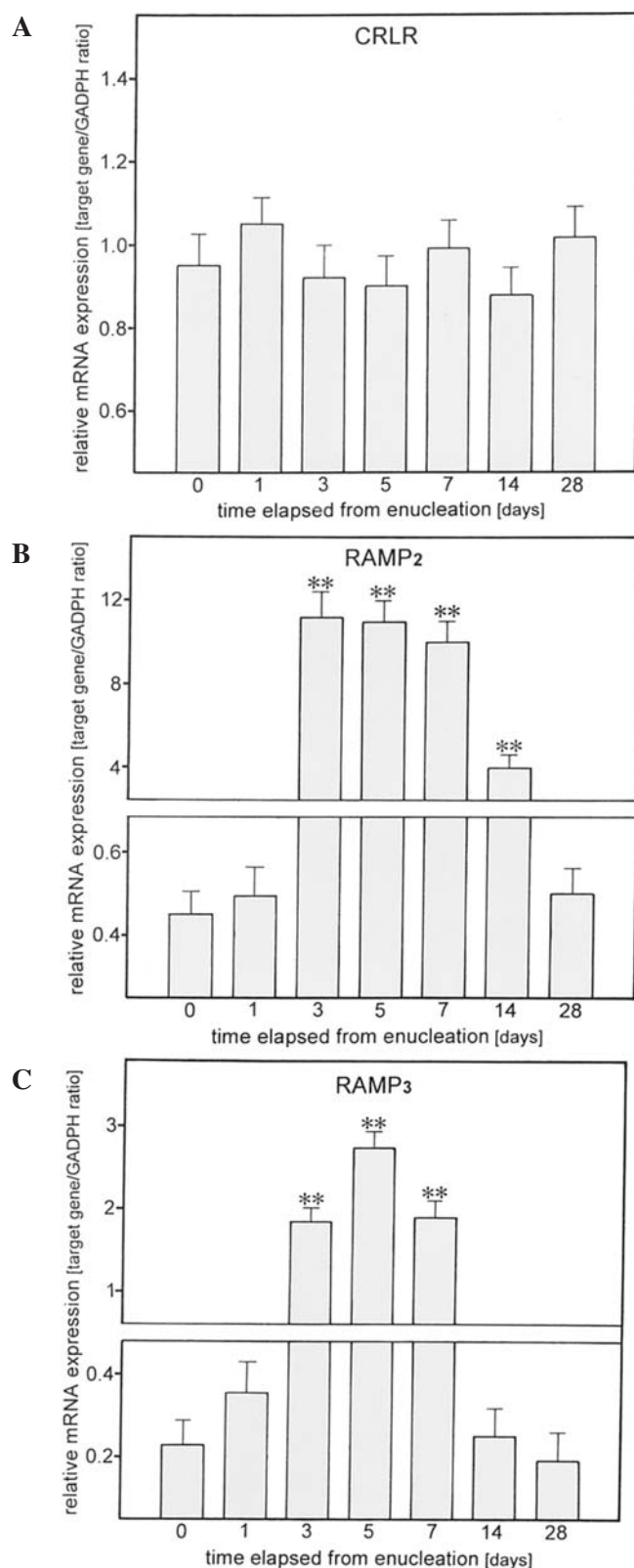


Figure 2. Semiquantitative real time-PCR assay of CRLR (A), RAMP2 (B) and RAMP3 (C) mRNA expression in regenerating rat adrenal cortex in relation to the time elapsed from enucleation and contra-lateral adrenalectomy. Bars are the means  $\pm$  SEM (n=4). \*\*P<0.01 compared to the controls (time 0).

the addition of elevated concentrations of AM was biphasic, peaking at 1 and 24 h, while in the regenerating adrenal cortex the response became manifest at 72 h, because it

conceivably ensued from the up-regulated translation of AM. AM increased the mRNA expression of RAMP2, but not RAMP3 in endothelial cells, while in the regenerating adrenal tissue the expression of both RAMPs was increased. It is possible that differences in the basal level of expression of the two RAMPs may account for this discrepancy. RAMP3 mRNA expression was found to be very low or absent in human umbilical vein endothelial cells (29; Albertin and Nussdorfer, unpublished data), but significant in rat and human ZG cells (11,12); hence, it seems legitimate to hypothesize that human endothelial cells are provided with functional AM<sub>1</sub>-R only, and ZG cells with both AM<sub>1</sub>-R and AM<sub>2</sub>-R. Finally, AM enhanced CRLR expression in endothelial cells, while up-regulation of CRLR transcription was not observed in regenerating adrenal tissue. It could be tentatively suggested that basal CRLR protein content in ZG cells, but not in endothelial cells, may be sufficiently elevated to face up-regulation of transcription/translation of RAMP2 and RAMP3 genes in giving rise to functional AM receptors. Accordingly, at time 0 the CRLR mRNA content of ZG stem cells was ~2- and 5-fold higher than that of RAMP2 and RAMP3, respectively.

Be that as it may, our present findings are in keeping with the view that AM up-regulates the expression of its own receptors; the concerted increase in AM production and AM receptor expression would greatly improve the autocrine-paracrine mechanism(s) by which this peptide favors proliferation of adrenocortical stem cells during gland regeneration. Obviously, optimal regeneration requires enhanced neovascularization to restore a normal vascular bed, and the up-regulation of the AM system may favor this process, because abundant evidence demonstrates the strong pro-angiogenic action of this peptide (30-33).

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