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Post-transcriptional regulation of CRLR expression during hypoxia

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Abstract

Adrenomedullin and CGRP are two potent vasodilator peptides, and their receptors are formed by heterodimerization of the CRLR and a RAMP molecule. Hypoxia is associated with many diseases of the cardiovascular system. It was recently shown that the human CRLR gene promoter contains an HIF-1α regulatory element, and that CRLR mRNA was increased by hypoxia in human endothelial cells. In the present work, we have assessed the effect of hypoxia on CRLR expression both in vivo and in vitro using two different experimental models. We have also investigated the effect of hypoxia on RAMP expression. (1) We analyzed the effects of a chronic hypobaric hypoxia on rat ventricle expression of RAMPs and CRLR. (2) Acute hypoxia was studied in human vascular smooth cells from coronary artery (CASMC) exposed for 6 h to 2% O_2. RT-PCR was used to analyze the mRNA expression, and protein levels were determined by Western blotting. A sharp increase in HIF-1α protein levels was induced by hypoxia in CASMC, and 3.5-fold rise of the CRLR protein occurred after 1 h of hypoxia in face of unchanged mRNA levels. The CRLR mRNA levels were only elevated later. A clear decrease of the CRLR protein level occurred after 3 and 6 h of hypoxia. Thus, acute hypoxia in CASMC induced a rapid change of the CRLR protein amount independently of changes in the CRLR mRNA. This finding suggested a major post-transcriptional effect of hypoxia on CRLR expression in CASMC. RAMP2 and adrenomedullin mRNAs were increased after 4 h, but no change was observed for RAMP1. Chronic hypoxia in rats enhanced both mRNA and protein levels of the three RAMPs and CRLR in right and left ventricles. Together, our in vivo and in vitro data suggested that hypoxia up-regulates both adrenomedullin and its receptor (CRLR/RAMP2) to enhance the signaling at the target cell.

Keywords: CRLR; RAMP; mRNA; Protein; Human coronary artery smooth muscle cell; Rat ventricle; Hypoxia; Hypobaric hypoxia

The cardiovascular effects of adrenomedullin and CGRP, two potent vasodilator peptides, are mediated through the G-protein coupled receptor calcitonin receptor-like receptor (CRLR) associated to receptor activity modifying proteins (RAMPs) [1]. Cell surface expression of CRLR and RAMP1 gave a functional CGRP receptor, whereas heterodimerization of CRLR with RAMP2 or RAMP3 formed an adrenomedullin receptor [2]. In human endothelial cells, the CRLR gene expression was regulated by hypoxia, and a regulatory element binding HIF-1α was found in the promoter [3]. If RT-PCRs have shown that CRLR mRNA levels were increased by acute hypoxia in human microvascular endothelial cells, however, the expression of the three RAMPs was unaffected by the low oxygen tension [3]. By contrast, in rat lungs, chronic hypoxia was reported to enhance mRNA levels for RAMP1 and RAMP3 [4].

We have in the present work assessed the effect of hypoxia on CRLR and RAMP expressions both at the mRNA and protein levels in two different experimental models. Rats exposed to a prolonged hypobaric hypoxia for three weeks were used to investigate in heart ventricles the CRLR and RAMP expressions during the

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course of hypoxia. In vitro, human coronary artery smooth muscle cells (CASMC) were subjected to acute hypoxia, and the relationship between changes in mRNA and protein levels for CRLR analyzed. Investigations were done at the mRNA level by semi-quantitative RT-PCR and at the protein level by Western blot analysis. Here, we provide new data indicating that acute hypoxia in CASMC induced rapid changes of the CRLR protein independently of alterations in its mRNA suggesting a major post-transcriptional effect, and that chronic hypobaric hypoxia was associated in rat heart ventricles with up-regulations of CRLR and RAMP proteins.

Materials and methods

Animals and experimental procedures. Male Wistar strain albino rats (IFFA CREDO, France), weighing approximately 180–200 g, were randomly assigned to two experimental groups: hypobaric hypoxia ($n = 68$) and control normoxic ($n = 35$). Animals were housed with a dark:light cycle of 12:12 h, at an ambient temperature of 22 ± 2 °C. They had free access to food and water. Experiments were performed in accordance with the Helsinki accords for human treatment of laboratory animals. Hypoxic animals were housed in a hypobaric chamber (Alsthom EY2400 Sauter, France) and exposed to a barometric pressure that was progressively reduced (20 min) until the equivalent of 5500 m altitude was reached (barometric pressure = 505 hPa; inspired partial oxygen pressure = 106 hPa). The chamber was opened every 2 days during 20–30 min to refill food and water dispensers. Rats were weighed to monitor body weight gain (BW). Hypoxic rats were randomly divided into 10 groups corresponding to different duration of exposure to hypobaric hypoxia. After the different periods of conditioning, animals were anesthetized with pentobarbital (Sanofi Santé Animal, Libourne, France) (70 mg/kg BW) administered intraperitoneally. The beating heart was then removed, washed in distilled water, and dissected. The atria were removed, the left ventricular free wall plus septum (LV) and right ventricular free wall (RV) were separated from excised hearts, weighed, and rapidly frozen in liquid N_2. All samples were stored at −80 °C until analyzes.

Cell culture. The human CASMC (PromoCell, Heidelberg, Germany) were maintained in smooth muscle growth medium supplemented with 5% fetal calf serum, 0.5 μg/L hEGF, 2 μg/L hFGF, 5 mg/L insulin, and a mixture of antibiotic (50 mg/L gentamicin + 50 μg/L amphotericin-B). Cultures were maintained at 37 °C with atmospheric air and 5% CO_2. The medium was changed every 48 h and cells were subcultured after treatment with trypsin and EDTA. Subconfluent human CASMC in their eighth passage were used for the experiments. For hypoxic stimulation, cells were placed in a modified incubator, flushed with a gas mixture containing 1%

Table 1
Oligonucleotides used for the amplification

Target	Sequence	Size (bp)	Annealing temperature (°C)	Number of cycles	Accession No.
rCRLR					
Sens (742–761)	5′-GCA GCA GAG TCG GAA GAA GG-3′	535	60	32	440339
Antisens (1274–1257)	5′-GCC ACT GCC GTG AGG TGA-3′				
rRAMP1					
Sens (364–384)	5′-AGC ATC CTC TGC CCT TTC ATT-3′	69	60	33	7592832
Antisens (431–412)	5′-CAG ACC ACC AGG GCA GTC AT-3′				
rRAMP2					
Sens (286–307)	5′-GCA ACT GGA CTT TGA TTA GCA G-3′	194	60	32	13928891
Antisens (479–461)	5′-GGC CAG AAG CAC ATC CTC T-3′				
rRAMP3					
Sens (81–101)	5′-GTA TGC GGT TGC AAT GAG ACA-3′	71	60	33	9910523
Antisens (151–131)	5′-ATC ATTTCGGCA AAG GCT TTC-3′				
rADM					
Sens (437–456)	5′-TTC GAG TCA AAC GCT ACC GC-3′	857	60	32	6978506
Antisens (1294–1275)	5′-ATC AGG CGC TCT CCA CCT TA-3′				
hCRLR					
Sens (471–490)	5′-ATG GAG AAA AAG TGT ACC CT-3′	361	60	28	U17473
Antisens (832–813)	5′-CAT GTT CTG TTG CTT GCT GG-3′				
hRAMP1					
Sens (82–100)	5′-TGG CCC ATC ACC TCT TCAT-3′	400	58	35	5032018
Antisens (481–462)	5′-GCC TAC ACA ATG CCC TCA GT-3′				
hRAMP2					
Sens (238–259)	5′-CGG TGA AGA ACT ATG AGA CAGC-3′	190	60	28	AJ001015
Antisens (428–409)	5′-GAT GAT CCT CTCTGC CAA GG-3′				
hRAMP3					
Sens (115–132)	5′-AGC AGA CAG GCA TGT TGG-3′	237	59	35	13928891
Antisens (352–335)	5′-CTG TCC ACG GTG CAG TTG-3′				
hADM					
Sens (262–283)	5′-CGA AAG AAG TGG AAT AAGTGG G-3′	205	60	25	D14874
Antisens (468–448)	5′-GTT CAT GCT CTG GCG GTA GCG-3′				
GAPDH					
Sens (244–264)	5′-ATC ACC ATC TTC CAG GAG CG-3′	573	60	20	M17701
Antisens (817–798)	5′-CCT GCT TCA CCA CCT TCT TG-3′				

O_2, 5% CO_2, and 94% N_2 (Air products, Strasbourg, France), and incubated at 37 °C up to 6 h. The tension level of O_2 was measured in situ during the course of hypoxia using a fyrite gas analyzer (Bacharach, New Kensington, PA, USA), and maintained at 2%. Control cells under normoxia were simultaneously used and placed in a different cell incubator.

RT-PCR. Total RNAs were isolated by the Trizol method (Invitrogen, Cergy-Pontoise, France). The RNA pellets were dissolved in sterile distilled water and quantified by optical density at 260 nm. cDNA were synthesized from 1 μg of total RNA. The reaction mixture had a final volume of 20 μL and contained 75 mM KCl, 50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM DTT, 20 U RNAsin (Promega), 200 U Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France), 1 mM of each dNTP (Promega, Charbonnière, France), and 0.25 μg of random primers (Amersham–Pharmacia Biotech). Annealing and primer extension were performed at 42 °C for 1 h. Then PCR was performed on a aliquot (2 μL) of this mix by adding 18 μL of a mix containing 22 mM Tris–HCl, pH 8.4, 55 mM KCl, 1.4 mM $MgCl_2$, 10 pmol of each specific primer (Invitrogen, Cergy-Pontoise, France), 2 nmol of each dNTP, 1 U *Taq* DNA polymerase (Invitrogen, Cergy-Pontoise, France), and 1 mCi [α-^{33}P]dCTP.

Semi-quantitative PCR was established by determination of the exponential phase of the PCR products. Thus, amplifications were routinely performed as shown in Table 1. The thermal cycling protocol was 94 °C for 45 s, 60 °C for 60 s, and 72 °C for 60 s. During the first cycle, the 94 °C step was extended to 5 min, and on final cycle the 72 °C step was extended to 5 min. Control reactions were carried out as previously described [5] in a T Gradient Thermocycler 96 (Biometra, Vysis, Voisins le Bretonneux, France). In some experiments, amplified products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide. The gel was fixed on 7% TCA for 30 min and then dried before direct exposition to Kodak Biomax autoradiographic (Kodak, France).

Western blots. The human CASMC and the rat ventricles were prepared by adding a membrane lysis buffer containing a detergent (16 mM CHAPS, 20 mM Tris–HCl, pH 7.5, 1 mM Na_2EDTA, and 1 mM DTT) with protease inhibitors (1 mM benzamidine, 1 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 0.5 mM PMSF (Sigma, St. Quentin Fallavier, France)) as already described [6]. The samples were then centrifuged at 15,000*g* for 30 min and the supernatants were collected. The protein content of the lysates was estimated using the Bradford method (Bio-Rad, France). The amounts of proteins used for Western blot analysis were 30 μg for human CASMC to detect HIF-1α, CRLR, and actin, and 20 μg to measure CRLR and RAMPs in rat heart ventricles. The proteins were loaded onto 4–20% SDS–Tris–glycine polyacrylamide gels and after electrophoresis the proteins were blotted onto PVDF membranes. The membrane was saturated for 2 h in TBS containing 0.1% Tween and 5% non-fat dried milk. A rabbit polyclonal antiserum, MR003, to the carboxy-terminus of rat CRLR was raised and affinity-purified according to a previously scheme [7,8]. The primary rabbit polyclonal antibodies against RAMP1 (sc-11379, Santa Cruz Biotechnology, Heidelberg, Germany), RAMP2 (sc-11380, Santa Cruz Biotechnology, Heidelberg, Germany), and RAMP3 (sc-11381, Santa Cruz Biotechnology, Heidelberg, Germany) were used at 1:500 dilution. A peroxidase conjugated rat anti-rabbit antibody (Sigma, St. Quentin Fallavier, France) was used at 1:15,000 dilution as secondary antibody. For identification of human CRLR, HIF-1α—an affinity-purified goat polyclonal antibody raised against the carboxy-terminus of the human CRLR (sc-18007, Santa Cruz Biotechnology, Heidelberg, Germany), and a mouse monoclonal antibody against HIF-1α (BD Transduction Laboratories, Le Pont de Claix, France), respectively, were used at 1:500 dilution overnight. The ECL Western blotting system (Amersham–Pharmacia Biotech., les Ulis, France) was used for detection. Autoradiographies were then scanned using a densitometer (Biocapt, Villmer Lourmat, Torcy, France) and the CRLR or RAMPs contents were normalized using actin.

Statistical analysis. For the RT-PCR experiments, all data are expressed as means ± SEM. A one-way variance analysis was used (SuperAnova, Abacus Concepts, USA) followed by the Duncan statistical test for mRNA levels during chronic hypobaric hypoxia. For Western blots, each value was represented with the corresponding median of the group, and statistical analysis was performed using the non-parametric Mann–Whitney *U* test (Statview v4.5, USA). For all treatments statistical significance was accepted at $P < 0.05$.

Results

mRNA levels in ventricles of rats exposed to a hypobaric hypoxia

Since GAPDH mRNA levels were unchanged during the period of hypoxia in both ventricles, results were expressed as the ratio of RAMP or CRLR mRNA level to the GAPDH mRNA level. All data were normalized by comparison of the ratio obtained for normoxic control values which was given as equal to 1. This expression of results similar to that recently reported by Nakanishi et al. [9] for adrenomedullin mRNA levels was useful to make direct comparisons between our work and the data of the Japanese group, because in both cases a similar schedule of hypobaric hypoxia was used. The adrenomedullin mRNA level was increased significantly (a 2-fold rise) at day 2 and day 4 in the RV (Fig. 1). A trend towards elevated values was observed at days 0.5 and 1 ($P > 0.06$). However, no change for adrenomedullin mRNA occurred in the LV during the 3 weeks of hypobaric hypoxia. The CRLR mRNA level increased transiently at day 14 in the RV, but in the LV, this increase was observed from day 14 to day 21 (Fig. 1). All three RAMPs increased in both ventricles, but with a different pattern during the course of hypoxia for each ventricle (Fig. 1). RAMP1 increased at day 2 and day 4 in the RV, but for RAMP2 the elevated levels were observed more later (i.e., between days 14 and 21); for RAMP3, peak values were observed at day 1 and day 14 in the RV. In the LV, RAMP1, RAMP2, and RAMP3 were significantly increased at day 15 (Fig. 1). A decrease in the mRNA level of RAMP1 was observed at day 18 and day 21 in the left ventricle.

CRLR and RAMP protein expressions in ventricles of hypobaric hypoxic rats

An increase in the expression of all RAMPs was detected during the course of the hypobaric hypoxia (Fig. 2) in both ventricles. Western blot analyzes of RAMP1, RAMP2, and RAMP3 showed that protein levels increased between days 4 and 25. Increases in the RV and in the LV were similar (a 3- to 4-fold increase). A more fine analysis of changes in RAMP1 protein showed that levels at day 0.5 were higher than those found at day 4 (data not shown). Significant changes in

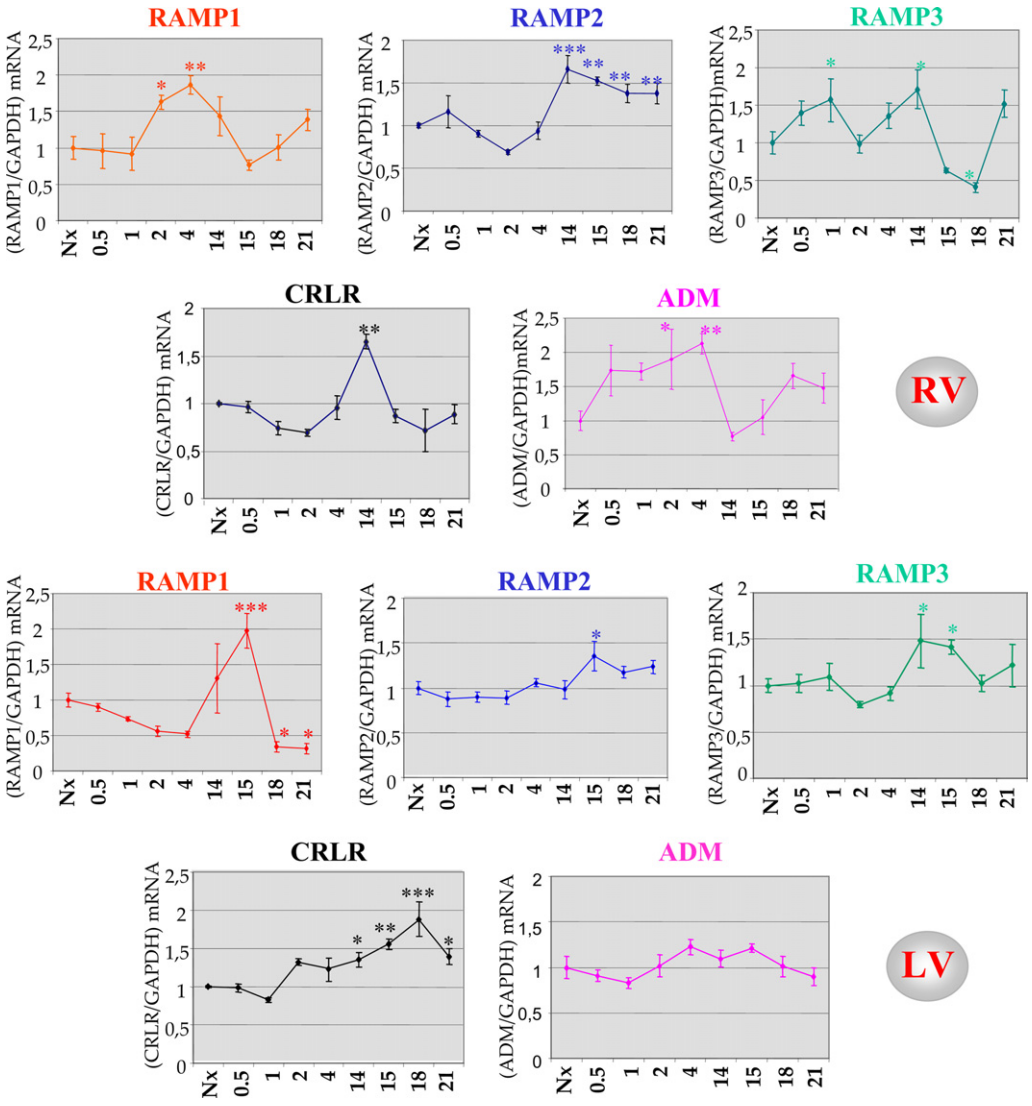

Fig. 1. Semi-quantitative RT-PCR data for adrenomedullin (ADM), CRLR, and RAMPS in hearts of hypobaric hypoxic rats. Right ventricle (RV), left ventricle (LV). Animals were exposed to 21 days of hypoxia and compared to normoxic (Nx) animals. To derive the data shown in this figure, the ratio between the xmRNA/GAPDH mRNA measured in Nx animals was expressed as equal to 1, and all other values for hypoxic rats were expressed in a relative manner. Means ± SEM of five animals per group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

the CRLR protein were observed for the LV (Fig. 2). When the results were expressed as the ratio between CRLR and actin used as a control probe, a significant increase was observed at day 18, but if data were not reported as a ratio, the rise in CRLR protein was already significant at day 15 (Fig. 2). A trend towards elevated values was observed at day 14 in the RV (data not shown) for CRLR expression, this change being significant when results were unrelated to the control actin probe.

Effects of acute hypoxia in CASMC

Hypoxia (2% O_2) in human CASMC induced increases in mRNA levels for adrenomedullin, CRLR, and RAMP2 (Fig. 3). Adrenomedullin mRNA was increased 4 h after hypoxia. Gene expressions for the molecular components of the adrenomedullin receptor (i.e., CRLR and RAMP2) also were increased. Compared to CASMC exposed to normoxia, the CRLR mRNA exhibited elevated values 3 and 4 h after hypoxia (a 2-fold increase at 4 h), whereas RAMP2 mRNA was only slightly increased at 4 h (Fig. 3). The mRNA level of RAMP1 was unchanged during the course of hypoxia (Fig. 3). RAMP3 mRNA was not measured because of the low level of expression in these experiments.

The effect of hypoxia in CASMC was assessed by the Western blot determination of HIF-1α; the specific monoclonal antibody used gave a single band around 120 kDa. The CRLR antibody revealed the presence of a specific immunoprecipitated band as a doublet around 60 kDa in total cell lysates from human CASMC

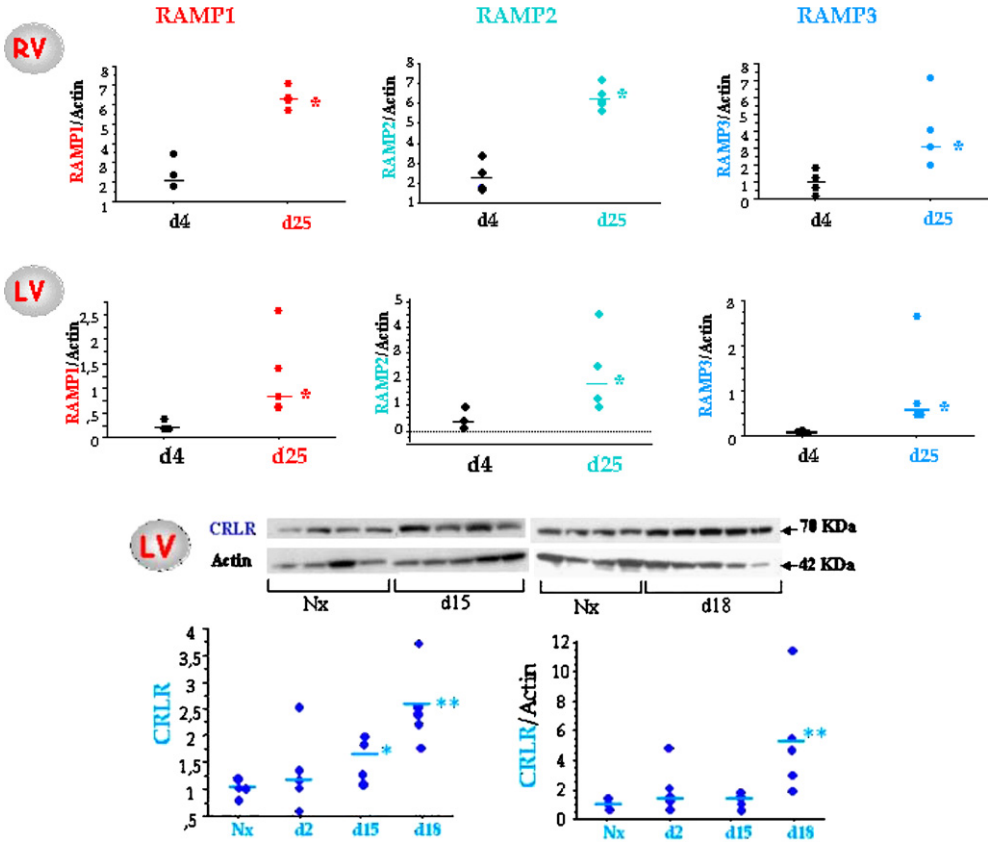

Fig. 2. Western blot determination of CRLR and RAMPs in rat ventricles during the course of hypobaric hypoxia. Right ventricle (RV), left ventricle (LV). The specificity of the induction was shown by the ratio between RAMPs or CRLR, and an actin control probe. Individual values and the medians were represented. $*P < 0.05$, $**P < 0.01$.

cultured in basal conditions; a shift in the size of the immunoreactive bands occurred following treatment with *N*-glycosidase-F, thus an apparent molecular weight of 45 kDa was observed for the single band of unglycosylated CRLR (data not shown). After application of hypoxia, the protein level of HIF-1α in CASMC was markedly increased from 1 to 6 h (Fig. 4). A rapid turnover of the CRLR protein was observed during the period of hypoxia since levels were increased 3-fold after 1 h of hypoxia and then fell in the following two hours (Fig. 4).

Discussion

Gene invalidation or gene delivery had shown that adrenomedullin and CGRP are of importance to the normal physiology of the cardiovascular system [10–12]. If the release of adrenomedullin and CGRP is altered in cardiovascular diseases, changes in the signaling of these two peptides through their receptors may be fundamental and characteristic of a pathology. As yet, few data were reported about changes in the protein expression of both CRLR and RAMPs. The lack of specific antibodies has impaired the development of such investigations. Therefore, our study is the first to characterize up-regulations at the protein level both for CRLR and RAMPs during acute and chronic hypoxia.

A transcriptional regulation of the CRLR gene in human microvascular endothelial cells by hypoxia was recently reported [3]. Lung endothelial microvascular cells exposed to 1% O_2 during 16 h showed an increase in adrenomedullin and CRLR mRNAs, but no change for the three RAMP mRNAs. Cloning of the proximal 5′-flanking region of the CRLR gene revealed the presence of one hypoxia responsive element, and promoter fragments were activated by hypoxia when transfected in primary microvascular endothelial cells [3]. We showed similar increase in mRNA levels of adrenomedullin and CRLR in hypoxic human CASMC. However, a slight increase in RAMP2 mRNA was observed in CASMC. The presence of one hypoxia-response element explained why human CRLR gene was regulated in vitro by hypoxia both in endothelial and smooth muscle cells. Unexpectedly, the CRLR protein level appeared regulated independently of the transcription in hypoxic human CASMC since protein amounts were 3.5-fold increase 1 h after hypoxia in spite of unchanged mRNA levels. Moreover, a marked fall of the CRLR protein occurred after 3 and 6 h of hypoxia in face of elevated

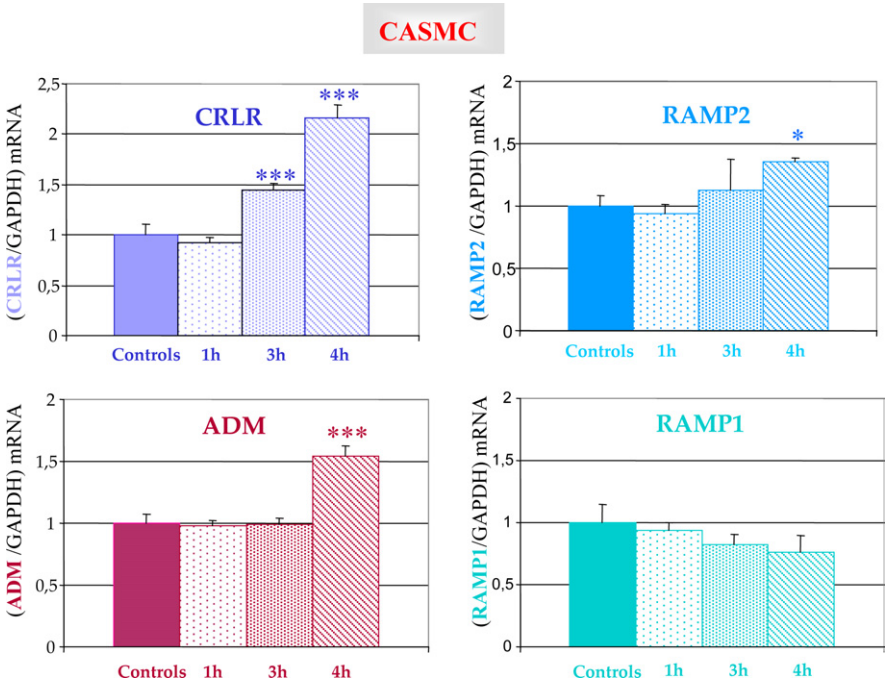

Fig. 3. Semi-quantitative RT-PCR data obtained during acute hypoxia of cultured human coronary artery smooth muscle cells (CASMC). The results were expressed as the ratio of the CRLR, RAMP, or adrenomedullin (ADM) mRNA to the GAPDH control probe. Means ± SEM of six cultures. $*P < 0.05$, $***P < 0.001$ from appropriate normoxic controls.

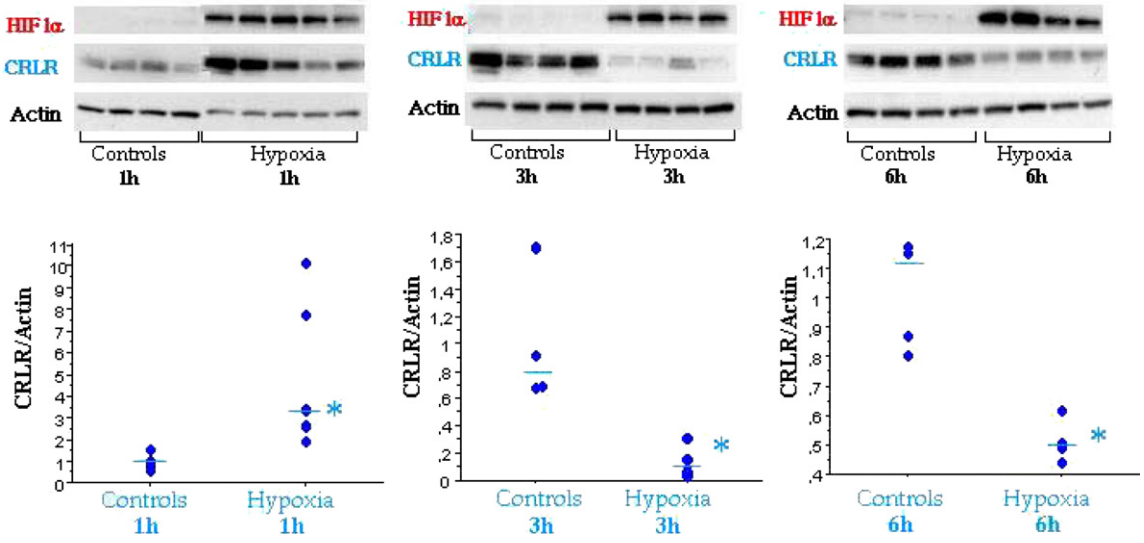

Fig. 4. Effects of acute hypoxia on CRLR expression in human coronary artery smooth muscle cells (CASMC). A sharp increase in HIF-1α expression was observed during the course of hypoxia (2% O_2). The CRLR protein was 3.5-fold induced after 1 h of hypoxia and then markedly decreased. Individual values were shown as well as the median. $*P < 0.05$ from appropriate normoxic controls.

levels of CRLR mRNA. These results emphasized rapid changes of the CRLR protein during acute hypoxia. Thus, it appeared that CRLR translation regulation was of major importance in comparison to the transcriptional regulation in hypoxic CASMC. The simultaneous up-regulations of CRLR, RAMP2, and its ligand adrenomedullin in vascular smooth cells might play a significant role in vascular responses to hypoxia to improve the tissue survival, this effect being strenghthened by a similar phenomenon described in endothelial cells [3].

In the present study, we also showed that chronic hypobaric hypoxia in rats induced up-regulations of both CRLR and RAMPs in heart. Our data for adrenomedullin mRNA were in accordance with previous findings of Nakanishi et al. [9]. Adrenomedullin mRNA was specifically induced in the RV since no change was

observed in the LV during the course of hypoxia, this observation being in favor of increased right ventricular workload on adrenomedullin gene expression. Hypobaric hypoxia is associated with increased pulmonary arterial pressure without alteration in systemic blood pressure [13], although both ventricles were exposed to hypoxia and neuroendocrine stimulation, only the RV was subjected to an increased hemodynamic load. Dynamic changes in the mRNA profiles for CRLR and RAMPs were observed during the 3 weeks of hypobaric hypoxia in both ventricles, but no effect of load was apparent for the RV. Up-regulations at the protein level were also observed for the three RAMPs and CRLR at the end of the period of hypobaric hypoxia. No effect of load on the RV protein expression of RAMPs and CRLR was found since amplitude responses of all RAMPs to chronic hypoxia were similar in both ventricles. The present study was in agreement with a previous work showing that chronic heart failure induced by aortic stenosis in the rat was associated with an increase in both the mRNA and protein levels of myocardial RAMP1 and RAMP3 [6]. In such a model, hypoxia was probably the cause of the up-regulations observed.

In conclusion, our observations made during chronic hypobaric hypoxia in rats suggested an up-regulation of CGRP and adrenomedullin receptors in ventricles because molecular components (CRLR and RAMPs) of such receptors were increased. Therefore, through its signaling at the receptor level, adrenomedullin may be more active at the target cell. If our results observed during acute and chronic hypoxia may be extrapolated to the hypoxia associated with myocardial ischemia, the cardioprotective effect of adrenomedullin [14] attributed to its antiapoptotic and antioxidative effects would be due, in part, to a consequence of the receptor up-regulation. This adaptation may be regarded in pathological conditions (heart failure) as a compensatory mechanism to prevent tissue and cell damage induced by the ischemia.

Acknowledgment

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