mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung

XIN QING, JOHN SVAREN, AND INGEGERD M. KEITH
Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Qing, Xin, John Svaren, and Ingegerd M. Keith. mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. Am J Physiol Lung Cell Mol Physiol 280: L547–L554, 2001.—Calcitonin gene-related peptide (CGRP) is a potent vasodilator. Our group has reported that exogenous CGRP may prevent or reverse hypoxic pulmonary hypertension in rats. The vasodilatory action of CGRP is mediated primarily by CGRP1 receptors. The calcitonin receptor-like receptor (CRLR) and the orphan receptor RDC-1 have been proposed as CGRP1 receptors, and recent evidence suggests that CRLR can function as either a CGRP1 receptor or an adrenomedullin (ADM) receptor. Receptor activity-modifying proteins (RAMPs) determine the ligand specificity of CRLR: coexpression of CRLR and RAMP1 results in a CGRP1 receptor, whereas coexpression of CRLR and RAMP2 or -3 results in an ADM receptor.

We used qualitative, semiquantitative, and real-time quantitative RT-PCR to detect and quantitate the relative expression of these agents in the lungs of rats exposed to normoxia (n = 3) and 1 and 2 wk of chronic hypobaric hypoxia (barometric pressure 380 mmHg, equivalent to an inspired O2 level of 10%; n = 3/time period). Our results show upregulation of RAMP-RAMP2 and RAMP3 mRNAs in hypoxic rat lung and no change in CRLR and RAMP2 mRNAs. These findings support a functional role for CGRP and ADM receptors in regulating the adult pulmonary circulation.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide generated from an alternatively spliced transcript of the calcitonin gene (2, 28). CGRP belongs to a superfamly of related peptides that consists of calcitonin, CGRP, islet amyloid polypeptide, and adrenomedullin (ADM). CGRP is predominantly expressed in the central and peripheral nervous systems (28, 31) and has diverse biological effects. In the lung, CGRP is abundant in neuroendocrine cells of the airway epithelium and is also found in sensory nerve fibers and intrapulmonary parasympathetic neurons (11, 13). CGRP is the most potent endogenous vasodilatory peptide discovered so far (38). In addition, our results showed that exogenous CGRP prevents the development of hypoxic pulmonary hypertension (HPH) and reverses existing HPH in rats (12, 32).

CGRP receptors have been characterized in a variety of tissues, mainly through functional binding assays. Specific CGRP binding sites have been demonstrated widely in both central and peripheral tissues including lung (19, 34). Several pharmacological and biochemical studies provide evidence for receptor heterogeneity, and at least three classes of CGRP receptors have been identified through studies with CGRP8–37 and [acetamido-
methylcysteine2,7]-CGRP ([Cys(ACM)2,7]-α-CGRP) (3, 27). The CGRP1 subtype is highly sensitive to the antagonistic properties of COOH-terminal fragments of CGRP, whereas CGRP2 possesses high affinity for the linear analog [Cys(ACM)2,7]-α-CGRP. Receptors that respond to both α-CGRP and salmon (but not human) calcitonin with high affinity are grouped together as a third type. The vasodilatory responses to CGRP are mediated primarily by CGRP1 receptors because the action could be potently antagonized by CGRP8–37 (35).

To date, cloning studies and functional assays have claimed several receptors to be CGRP1 receptors. First, the canine orphan receptor RDC-1 was originally cloned from dog thyroid cDNA by using the PCR with degenerate primers that correspond to consensus sequences of transmembrane domains 3 and 6 of other G protein-linked receptors (17), and RDC-1 has been identified as a CGRP1 receptor (10). Second, a calcitonin receptor-like sequence [calcitonin receptor-like receptor (CRLR)] was initially cloned in rat lung (24), and its human homolog has been reported to be a CGRP1 receptor (1). Most recently, receptor activity-modifying proteins (RAMPs) were cloned; their biological functions are transporting CRLR to the cell membrane, determining its glycosylation state, and defining its pharmacology (22). Coexpression of RAMP1 and CRLR was found to create novel CGRP1 receptors in cell lines, whereas RAMP2 or RAMP3 presents CRLR at the cell surface as an ADM receptor (6, 22).

To better understand the mechanisms of the pulmonary vasodilatory activity of CGRP in HPH, we exam-
ined the mRNA expression of these CGRP1/ADM receptor-related genes in rat lungs during normoxia and chronic hypoxia by use of qualitative, semiquantitative, and real-time quantitative RT-PCR.

**MATERIALS AND METHODS**

*Animal treatments.* Adult male Sasco Sprague-Dawley rats weighing 175–200 g were randomly assigned to groups (3 rats/group) in which they were exposed to normoxia or 1 or 2 wk of hypoxia. Each rat was placed unrestrained in a separate cage in ambient room air (normoxia) or in a hypobaric hypoxia chamber with a barometric pressure of 380 mmHg, equivalent to an inspired O2 level of 10%, ambient humidity, and ambient room air CO2 level (Biotron, University of Wisconsin-Madison). The 1-wk hypoxia-treated rats were housed in room air (normobaric normoxic) for 1 wk before hypoxic exposure to ensure that all rats were the same age at the end of the study. The hypoxic chamber was opened twice a week to clean cages and to replenish food and water. The maximum duration of time that the hypoxic chamber was opened each time was 30 min. The normoxic rats were housed under similar but normobaric normoxic conditions. Food and water were given ad libitum. The rats were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-certified facilities and treated humanely according to protocols approved by the Animal Resources Center and the Graduate School of the University of Wisconsin-Madison.

At the end of the hypoxic exposure, the rats were transferred to the laboratory and placed in a normobaric chamber under a continuous flow of 10% O2 in N2 before tissue was collected. Each rat was removed from the chamber and deeply anesthetized with pentobarbital sodium (80 mg/kg ip). The superior mesenteric artery was cut to drain the blood, and the lungs were rapidly isolated and immediately flash frozen in liquid nitrogen and stored at −70°C.

*Total RNA extraction and DNase digestion.* Total RNA was isolated from 30 mg of the tissue at the peripheral region of the lungs with the use of an RNasey Minikit (Qiagen, Valencia, CA). Before RT-PCR was performed, samples were pretreated with DNase (RQ1 RNase-free DNase; Promega, Madison, WI) according to the instructions provided by the manufacturer.

*Oligonucleotides (primers) and RT-PCR.* The primers for CRLR, RDC-1, RAMP1, and RAMP3 were designed based on published cDNA sequences of rat CRLR, rat RDC-1, rat RAMP1, and rat RAMP3 (GenBank accession nos. AJ001015) because only the human sequence was available for RAMP2 at the time this study was undertaken. These were upstream primer 5'-CTG GGC GCT GTC CTG AAT C-3', corresponding to nucleotides 159–177 and downstream primer 5'-GAG AAG GTG GCC TGC ACC A-3', corresponding to nucleotides 484–466. According to the human mRNA sequence of RAMP2, the expected length of the amplified DNA fragment should be 326 bp.

DNase-pretreated total RNA was reverse transcribed and amplified by PCR with a one-step RT-PCR kit (Access RT-PCR system; Promega) in a total volume of 50 μl with 0.2 μg of total RNA and 1 μM each upstream and downstream specific primer corresponding to the sequence of interest. Reactions were incubated at 48°C for 45 min, heated to 94°C for 2 min, and cycled according to the following parameters: 94°C for 30 s (denaturation), 55°C for 1 min (annealing), and 68°C for 2 min (extension) for a total of 40 cycles. A 7-min final extension at 68°C was performed after 40 cycles. Each RT-PCR was repeated at least once to ascertain reproducibility. Negative control, positive control, and “no-RT” control reactions were performed. Negative controls were run in which the RNA templates were replaced by nuclease-free water in the reactions. For the positive control reaction, we used 2.5 amol or 1 × 106 copies of the supplied positive control RNA (1.2-kb kanamycin resistance gene mRNA) with carrier (Escherichia coli rRNA) and the upstream and downstream control primers (final concentration 1 μM in 50 μl).

The amplification product obtained from the positive control reaction should be 323 bp long and an ~220-bp amplification product may be observed, which arises from the amplification of a sequence in the carrier RNA. For a no-RT control, nuclease-free water was used in place of the reverse transcriptase. In the RAMP2 study, we used lung tissue samples from normoxic and 2-wk hypoxia-treated rats only, and the RT-PCRs were performed as above.

Ten microliters from each RT-PCR product were loaded on a 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide and separated by electrophoresis. DNA ladders were run in the outside lane to confirm the molecular sizes of the amplified products. To verify the identity of the RT-PCR products, the major products were excised from the agarose gels and purified with the Wizard PCR Preps DNA purification system (Promega) and then subjected to sequence analysis (Biotechnology Center, University of Wisconsin-Madison). RT-PCR products were directly purified and sequenced by the Biotechnology Center if agarose gel electrophoresis showed a single bright band.

*Semiquantitative RT-PCR.* To evaluate these CGRP1 and ADM receptor mRNA expression levels semiquantitatively, 4 μg of DNase-pretreated total RNA were reverse transcribed with random primers in 80-μl reactions with a commercially available kit (Reverse Transcription System, Promega). Reactions were incubated at room temperature for 10 min, at 42°C for 1 h, at 99°C for 5 min, and at 0–5°C for 5 min and then stored at −20°C until use. PCR amplification of each gene product was carried out in parallel 25-μl reactions using PCR Core Systems I (Promega) with 1 μM of the aforementioned specific forward and reverse primers (the primer concentration for RAMP3 amplification was 0.2 μM) and 1 μl of RT product (2 μl of RT product was used for RAMP3). The mixed samples were placed at 95°C for 2 min and then cycled as follows: 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min. A final extension step of 5 min at 72°C was carried out. The 18S rRNA was used as an internal control for sample loading. The upstream primer (5'-CCG CAG CTA GGA ATA ATG
respectively), and the rat RAMP2 partial cDNA sequence we discovered (see Fig. 5).

Table 1. Primers and probes for real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Position, nucleotides</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRLR</td>
<td>Forward primer 5’-CAA TGG CTT TTC CCA CTC TGA-3’</td>
<td>1899–1919</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5’-GCA CAT GGC TGA TTG ACA-3’</td>
<td>1966–1946</td>
<td></td>
</tr>
<tr>
<td>RDC-1</td>
<td>Forward primer 5’-GGG ACG ACA AGG TCA GGA A-3’</td>
<td>685–703</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5’-GGA CAG CAA AAC CCA AGA TGA-3’</td>
<td>753–732</td>
<td></td>
</tr>
<tr>
<td>RAMP1</td>
<td>Forward primer 5’-ATC ATT TCG GCC GCA AGG ACA-3’</td>
<td>81–101</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5’-ATC ATT TCG GCC GCA AGG CCT-3’</td>
<td>151–131</td>
<td></td>
</tr>
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The primers and probe were designed based on the published cDNA sequences of rat calcitonin receptor-like receptor (CRLR), rat RDC-1, rat receptor activity-modifying protein (RAMP) 1, rat RAMP3 (GenBank accession nos. L27487, AJ010828, AB028933, and AB030944, respectively), and the rat RAMP2 partial cDNA sequence we discovered (see Fig. 5).
By RT-PCR, the primers for RDC-1 amplified a product of the expected size (354 bp) that corresponded to rat mRNA encoding RDC-1 from each of the three samples (Fig. 2). Sequence analysis of this product indicated 100% homology with rat RDC-1 cDNA. Similarly, the primers for RAMP1 amplified a 230-bp product that corresponded in size to the RT-PCR product expected from rat RAMP1 mRNA (Fig. 3). Sequence analysis performed on the product showed 100% homology with rat RAMP1 cDNA.

The RAMP2 primers amplified five bands from both normoxic and hypoxic total RNA samples. Their sizes were ~500, 375, 325, 275, and 225 bp (Fig. 4). Sequence analysis was performed on the five products and revealed that the ~325-bp band had a high degree of identity with human and mouse RAMP2 cDNA (Fig. 5), suggesting that this is the rat counterpart of RAMP2. The sequence data reported in Fig. 5 contain only 186 bp to avoid including internal unsequenced spacers. This sequence was submitted to the GenBank/European Molecular Biology Laboratory database (accession no. AF162778). A new RAMP2 primer set was designed based on the sequence discovered (Fig. 5), and RT-PCRs were repeated with the new primers using methods described in MATERIALS AND METHODS. A single band of 175 bp was found in all samples (Fig. 6). Sequence analysis confirmed its identity. The new primer set was used for the semiquantitative RT-PCR of RAMP2 described in Semiquantitative and real-time quantitative RT-PCR to compare the rat lung RAMP2 expression levels during normoxia or after chronic hypoxia.

A band of 416 bp was amplified from each of the three samples with RAMP3 forward and reverse primers (Fig. 7). Sequence analysis performed on the product indicated that it was amplified from rat RAMP3.

No band was detected in negative controls and in no-RT controls, whereas a strong band of 323 bp and a weaker band of ~220 bp were observed in the positive control as expected.

**Semiquantitative and real-time quantitative RT-PCR.** After completion of the initial qualitative analysis that provided information on the existence of transcripts, it was necessary to quantify the levels of CRLR, RDC-1, and RAMP1, -2, and -3 transcripts in normoxic and hypoxic rat lungs to further understand these receptors and RAMPs during normoxia and hypoxia. Semiquantitative RT-PCR (see Fig. 8) demonstrated that RAMP1 and RDC-1 mRNA expression were increased in the lungs of 1-wk and 2-wk hypoxia-treated rats when compared with the results in normoxic rats. In contrast, no significant change in mRNA expression was found for CRLR or RAMP2. Moreover, there was a trend of increasing RAMP3 mRNA with hypoxia, although the change was inconsistent because of the high variance of RAMP3 mRNA expression between individual samples. The 18S rRNA was used as the internal control for cDNA quantity and quality and was compared across all normoxic and hypoxic lungs.

Preliminary experiments demonstrated that 18S rRNA did not change with 2 wk of exposure to the level of hypoxia used in this study, whereas other housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase and β-actin, increased (data not shown). In this study, approximately equivalent amounts of 18S rRNA were shown in each sample.

Quantitative estimates of the relative abundance of CRLR, RDC-1, and RAMP1, -2, and -3 mRNAs were also obtained with real-time quantitative RT-PCR after normoxic or hypoxic treatments (Fig. 9). With the use of SYBR Green I assays, three- and twofold up-
CGRP1 AND ADRENOMEDULLIN RECEPTOR mRNAs IN RAT LUNG

regulation of RAMP1 mRNA was detected after 1- and 2-wk hypoxic treatments, respectively, whereas CRLR and RAMP2 mRNA expression were unchanged. RAMP3 mRNA was significantly increased by hypoxia, and there was a highly significant linear correlation between the amounts of mRNA and time spent in hypoxia ($r = 0.904; P = 0.000$) with individual normalized means in the data set (Fig. 10). Moreover, RDC-1 mRNA expression was upregulated twofold after hypoxia, although the change was not significant ($P = 0.10$ by ANOVA). By using a prelabeled fluorescent probe (the fluorogenic $5'$$3'$-nuclease assays), similar results with a very small SE were obtained for RAMP1, which suggests higher sensitivity. The 18S rRNA, which was used as an internal control, was unchanged by hypoxia according to both the fluorogenic $5'$$3'$-nuclease and SYBR Green I assays.

DISCUSSION

CGRP is well known for its vasodilatory action via CGRP1 receptors located on vascular endothelium and smooth muscle (18, 36). Reversal of HPH by exogenous CGRP has been previously reported in rats (32), and the protective role of endogenous CGRP in HPH has been shown (33). Although specific CGRP binding sites have been reported in the lung, little is known about the expression of these novel CGRP1 receptors. Our previous study revealed that CRLR, RDC-1, and RAMP1 mRNAs were expressed in the rat lungs in normoxia and chronic hypoxia. We were not surprised to detect the expression of CRLR mRNA in the rat lungs because previous studies (1, 5, 24) have demonstrated a high level of CRLR mRNA expression in both human and rat lungs. In the original study of RDC-1, Northern blot analysis identified the heart and kidney as the major expression sites of RDC-1 mRNA, with weaker signals in the brain and spleen and no signal in the stomach, liver, lung, or salivary glands (10, 17). To our knowledge, the present study is the first demonstration of RDC-1 mRNA expression in the lung. This discovery is probably a result of the higher sensitivity of the RT-PCR method compared with Northern blot analysis.

RAMP1 mRNA was not detected in human lung by Northern blot analysis (6, 22). However, RAMP1 mRNA was expected to be expressed in lung at a low level (22). This, therefore, led us to investigate its expression in rat lung by RT-PCR. Our study clearly indicates that RAMP1 is expressed in rat lung. According to the RAMP theory, CRLR can function as either a CGRP1 receptor or an ADM receptor, with receptor specificity determined by a RAMP family; RAMP1 transports CRLR to the plasma membrane as a CGRP1 receptor, whereas RAMP2 and RAMP3 present CRLR at the cell surface as an ADM receptor (6, 22). The discovery of RAMP1 expression in lung supports the RAMP theory and may explain the reversal of HPH by exogenous CGRP (32).

ADM is structurally and functionally related to CGRP. Human ADM is a 52-amino acid polypeptide (14), and rat ADM has 50 residues (29). ADM shares slight amino acid sequence similarity with CGRP (24% identity in all 3 species).
in humans and 27% in rats). However, both ADM and CGRP have a six-residue ring structure formed by an intramolecular disulfide bridge between two cysteine residues, and both also have the COOH-terminal amide structure. These important structural similarities contribute to overlapping biological effects between CGRP and ADM. The main physiological effect of ADM reported thus far is its vasodilatory property, which is second only to that of CGRP (37). In certain vascular beds, the vasodilator action of ADM is thought to be mediated at least in part by CGRP1 receptors because CGRP8–37 selectively inhibits the vasodilator responses of ADM (4, 16, 25). However, it is also possible that ADM acts via its own receptor in some vascular beds (7, 9, 23). These observations seem to be consistent with the RAMP hypothesis (30). ADM can reduce hypoxia-induced pulmonary hypertension in the rat lung, and part of the response to ADM is thought to be mediated by CGRP1 receptors (39). However, the role of ADM is believed to be most important in the late fetal period (21).

By Northern blot analysis, ADM mRNA was found to be highly expressed in several tissues, including adrenal medulla, heart, lung, and kidney, in both rats and humans (15, 29), and immunoreactive ADM has also been detected in the plasma (8). Specific ADM binding sites have been reported in many tissues, including heart and lung (26). Our present RT-PCR analysis clearly demonstrated that RAMP2 and -3 mRNAs are expressed in rat lungs both in normoxia and after hypoxic treatment. These data are consistent with the localization of specific ADM binding sites and further support the RAMP theory. Interestingly, RAMP3 is expressed strongly in the human lung by Northern blot analysis (22), whereas it is only just detectable by RT-PCR in rat lung in the present study, which suggests that RAMP3 may not be very important in rat lung.

Using semiquantitative RT-PCR, we found upregulation of RDC-1, RAMP1, and RAMP3. To obtain more detailed information, we performed real-time quantitative RT-PCR assays that showed a higher sensitivity than our conventional semiquantitative RT-PCR. Results of real-time quantitative RT-PCR were in agreement with those obtained from semiquantitative RT-PCR. A two- to threefold increase in RAMP1 mRNA was detected after chronic hypoxia by both the fluoro-
genic 5′-nuclease assays and SYBR Green I assays. However, the fluorogenic 5′-nuclease assays tend to be somewhat more sensitive for detection of low amounts of target such as RAMP1 mRNA because the use of fluorogenic probes avoids the complications caused by detection of nonspecific amplification, which is more of a problem at low target levels. Although use of the fluorogenic probe may provide higher specificity and sensitivity, we decided to use SYBR Green I assays in the present study because of the high cost of the fluoro-
nently labeled TaqMan probes.

Radioligand studies indicated that CGRP binding capacity in the vascular endothelium was significantly elevated after 5 days of hypoxia (means ± SE: control 4.6 ± 0.4 vs. hypoxic 16.6 ± 2.4 amol/mm²) (20). This may reflect less endogenous binding (and more free receptors) induced by the suppression of pulmonary CGRP release (32). Alternatively, receptor upregulation could contribute to the increased radioligand binding. Our present semiquantitative and real-time quanti-
tative RT-PCRs revealed the regulation of the expression of these receptors after hypoxic exposure. Both RAMP1 and RDC-1 mRNA expression were sig-
nificantly increased, which supported the CGRP1 receptor upregulation hypothesis. The increase in RAMP1 mRNA and unchanged CRLR mRNA in hyp-
oxia suggest that CGRP reception at the CRLR is primarily upregulated by RAMP1.

To better understand these related receptors and their roles during normoxia and hypoxia, knowledge of their precise localization in lung tissue would be useful. However, it is impossible to localize most of these mRNAs by in situ hybridization only, due to the small amounts of RDC-1, RAMP1, and RAMP3 mRNAs in the rat lung (10, 17, 22).

In summary, our present study demonstrates that CRLR, RDC-1, RAMP1, RAMP2, and RAMP3 mRNAs are expressed in normoxic and chronic hypoxic rat lungs. It supports previous reports showing specific CGRP and ADM binding sites in the lung. Moreover, upregulation of RAMP1, RAMP3, and RDC-1 mRNA expression with hypoxia is revealed. These findings, as well as the partial cDNA sequence of rat RAMP2, should benefit further studies on these CGRP1 and ADM receptors.

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