Adrenomedullin expression and function in the rat carotid body

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Abstract

Adrenomedullin (AM) immunoreactivity has been found in granules of the glomus (type I) cells of the carotid bodies in rats. The identity of these cells was ascertained by colocalization of immunoreactivities for AM and tyrosine hydroxylase in their cytoplasm. Exposure of freshly isolated carotid bodies to synthetic AM resulted in a concentration- and time-dependent degranulation of glomus cells as measured by dopamine (DA) release. DA release reached a zenith 30 min after exposure to AM (94.2% over untreated controls). At this time-point, the response to AM was similar to the one elicited by 5 min of exposure to 100 mM K⁺. Nevertheless, injection of 1 µl

60 nM AM/g body weight into the tail vein of the rats did not induce statistical differences in DA release from the carotid bodies. Exposure of the oxygen-sensitive cell line PC-12 to hypoxia elicited an increase in AM mRNA expression and peptide secretion into serum-free conditioned medium. Previous data have shown that elevation of AM expression under hypoxia is mediated through hypoxia-inducible factor-1, and that exposure of chromaffin cells to AM results in degranulation. All these data suggest that AM is an important autocrine regulator of carotid body function.

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Introduction

Adrenomedullin (AM) is a 52 amino acid peptide containing an internal disulfide bond and a C-terminal amide group. It was originally isolated from a pheochromocytoma and shown to elevate cAMP in platelets (Kitamura *et al.* 1993). Many functions have been ascribed to AM. This peptide can act as a vasodilator, bronchodilator, growth factor, regulator of hormone secretion, neurotransmitter, antimicrobial agent, and a controller of renal function, among others (Hinson *et al.* 2000, López & Martínez 2002).

The most characteristic activity of AM is the induction of an intensive, long-lasting dose-dependent hypotension in humans, rats, rabbits, dogs, cats, and sheep. AM dilates resistance and capacitance vessels in the kidneys, heart, brain, lung, hindlimbs, and mesentery. Moreover, AM elicits relaxation of ring preparations of the aorta and cerebral arteries. An intravenous injection of human AM to conscious sheep causes a dose-dependent fall of blood pressure, an increase in heart rate and cardiac output with a small reduction in stroke volume, as well as a marked decrease in total peripheral resistance (Parkes 1995). In human coronary arteries, AM elicits vasodilatation through specific AM receptors and by enhancing production of nitric oxide (Terata *et al.* 2000). AM levels increase under

pathological conditions such as heart failure, myocardial infarction, hypertension, and septic shock (Eto *et al.* 1999). Conversely, intracerebroventricular administration of AM induces hypertension and tachycardia in conscious rats (Saita *et al.* 1998). In addition, when microinjected into the area postrema, AM increases blood pressure and heart rate (Mark *et al.* 1997). These observations thus indicate that AM influences blood pressure in a differential manner depending on the site of action.

Carotid bodies are a small pair of highly vascularized and well-perfused organs located at each carotid artery bifurcation. They are sensory organs that detect decreases in arterial blood oxygen and react by stimulating the nucleus tractus solitarius in the brain stem. This nucleus can precipitate a wide array of systemic reflex responses, including ventilatory and cardiovascular acclimatization. A bulk of evidence suggests that glomus (type I) cells are the initial site of transduction and that they release transmitters in response to hypoxia, which in turn cause depolarization of nearby afferent nerve endings. Acute hypoxia causes a rapid response within milliseconds, which is mediated by oxygen-sensitive K⁺ channels located in the glomus cell membrane. On the other hand, responses to chronic hypoxia are much slower and include changes in gene expression profiles that are responsible for the phenotypic changes observed in pathophysiological situations such as

sleep apneas, apneas in premature infants, asthma, or pulmonary fibrosis. Some of these hypoxia-induced changes in gene expression include elevation in the levels of dopamine (DA), norepinephrine, nitric oxide, and endothelin, and a decrease in the levels of substance P (López-Barneo *et al.* 2001, Prabhakar *et al.* 2001).

AM is expressed by chromaffin cells of the adrenal medulla (Kapas *et al.* 1998), which share a common embryonic origin with glomus cells and have similar mechanisms of oxygen sensing and response (Millhorn *et al.* 1996) and are often used as physiological models for carotid body studies. AM expression is upregulated under hypoxic conditions through hypoxia-inducible factor-1 (HIF-1) (Garayoa *et al.* 2000) and has been found in many structures of the central nervous system (Serrano *et al.* 2000). In addition, exposure of the rat brain to ischemia reperfusion results in a dramatic increase in AM expression in both neural and vascular structures of the brain (Serrano *et al.* 2001). Based on these facts we decided to study whether AM is expressed in the carotid body and, if so, whether it influences carotid body physiology.

Materials and Methods

Animals and surgical procedure

Experiments were performed using carotid bodies from adult male Wistar rats (body weight (BW) 240–280 g) which were housed under standard conditions of 50% humidity, 16 liters/min air exchange, and a 12 h light:12 h darkness cycle, with food and water provided *ad libitum*. Animals were anesthetized by intraperitoneal injection of 80 mg/kg ketamine (Ketolar; Park-Davis, Madrid, Spain) and 10 mg/kg xylazine (Rompum; Bayer, Madrid, Spain). Carotid bodies were removed from the carotid bifurcation, cleaned of surrounding connective tissue and either fixed for immunodetection (see below) or treated as indicated and kept frozen at -80°C until extracted. All procedures were approved by the animal ethics committee of the University of Navarra.

Double immunofluorescence and confocal microscopy

Carotid bodies from four animals were fixed in 4% paraformaldehyde for 30 min, embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and frozen in liquid nitrogen. Sections (20 μm thick) were blocked with a mixture of 3% donkey and 3% goat normal sera (Jackson ImmunoResearch Labs, West Grove, PA, USA) and then exposed to a mixture of two primary antibodies obtained from different species, overnight at 4°C . The mixture consisted of a previously characterized rabbit antibody against AM (Martínez *et al.* 1995) and a monoclonal mouse antibody against tyrosine hydroxylase (Chemicon, Temecula, CA, USA), both at a final

concentration of 1:1000. The next day, the sections were incubated for 1 h with a mixture of Bodipy-goat anti-rabbit (Molecular Probes, Eugene, OR, USA), and Texas red-donkey anti-mouse (Jackson ImmunoResearch Labs), both at a final concentration of 1:200. Counterstaining with DAPI (Molecular Probes) was performed for 10 min. After thorough washes, the slides were mounted in GelMount (Biomed, Foster City, CA, USA) and observed with a Zeiss laser scanning microscope 510, equipped with four lasers.

Analysis of DA secretion

Freshly isolated carotid bodies (12–16 per treatment) were incubated with synthetic AM (Peninsula Labs, San Carlos, CA, USA) in a time-course (5–60 min) and a dose-response (10^{-8} to 10^{-5} M) manner. Individual carotid bodies were immersed in 100 μl 0.4 M perchloric acid, 1 mM EDTA, and 0.1% sodium metabisulfite and vortexed for 1 min to extract DA as described (Verbiese-Genard *et al.* 1983). DA contents in the extraction samples (20 μl) were determined using high-performance liquid chromatography with electrochemical detection as previously reported (Kilts *et al.* 1981). The mobile phase composition was 0.1 mM Na_2HPO_4 , 0.1 M citric acid, 0.74 mM octansulphonic acid, 1 mM EDTA, and 14% methanol, pH 3.4. DA contents were calculated by extrapolation against a standard curve (125–500 pg/20 μl DA). A group of 12 carotid bodies was incubated with 100 mM KCl for 5 min, to check the validity of our experimental procedure by studying the response of the preparation to a well-known depolarizing stimulus (Rosenbland & Nilsson 1993). Different sets of samples were used for each incubation time.

For the *in vivo* study, 18 animals received 1 μl 60 nM AM/g BW intravenously. After 30, 60, and 90 min of the injection, six rats per time-point were anesthetized as before and the carotid bodies dissected out. DA content analysis was performed as for the *in vitro* experiments. In one control group ($n=6$), the carotid bodies were dissected immediately after being injected with saline. To take into account variations due to stress after injections, a second group of controls ($n=6$) were obtained from animals killed 90 min after the saline injection.

Cell culture and Northern blot analysis

Cell line PC-12 has been previously characterized as an oxygen-sensing cell line and has been frequently used as a model for carotid body function (Millhorn *et al.* 1996). This rat cell line was obtained from ATCC (Manassas, VA, USA) and grown in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA). Freshly fed cells were equally distributed into ten T-175 culture flasks and maintained until 50% confluent. At this time, five flasks

were kept under normoxic conditions (20% O₂, 5% CO₂, 75% N₂), whereas the other five were subjected to hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) in a hypoxia chamber as previously described (Garayoa *et al.* 2000). Total RNA was extracted at 0, 4, 8, 12, and 24 h from both environments using the guanidine isothiocyanate and cesium chloride method. Total RNA (15 µg) was loaded per lane in 1% agarose gels containing 2.2 M formaldehyde, blotted by capillarity onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH, USA), and baked for 2 h at 80 °C. Equal loading and integrity of RNA were monitored by ethidium bromide staining of the 18 S subunit of rRNA.

A rat-specific AM (GenBank accession number U15419) probe was prepared by PCR with primers: sense (rAM_{43–66}) 5'-CTC GGC TTC TCA TCG CAG TCA GTC-3' and antisense (rAM_{1211–1234}) 5'-CAC ACG GGG AAC CAA ACA ACC TTA-3'. The 1192 bp PCR product was cloned into pCR II (Invitrogen, Carlsbad, CA, USA) and sequenced to ensure sequence identity. The probe was excised from the vector using EcoRI and labeled with [α -³²P]dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA, USA) by random priming and unincorporated nucleotides were removed by Probe-Quant G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was carried out overnight at 42 °C in a hybridization buffer containing 40% formamide. After stringency washes, blots were exposed to XAR film (Eastman Kodak, Rochester, NY, USA) at –80 °C for varying times. These experiments were repeated three times.

Reverse transcription and real-time PCR

To quantify variations in the levels of AM mRNA induced by hypoxia, we performed real-time PCR on the same samples analyzed by Northern blotting. Total RNA (3 µg) from treated PC-12 cell samples was retro-transcribed in a final volume of 25 µl using SuperScript First-Strand Synthesis system (Invitrogen) following the manufacturer's instructions.

The quantitative real-time PCR reaction was run in an Opticon cycler (MJ Research, Waltham, MA, USA) using Sybr Green PCR master mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Thermocycling was performed in a final volume of 25 µl containing 2 µl cDNA (1:10 dilution) and 400 nM primers (primers for AM as above or β -actin forward: 5'-ACA ATG AGC TGC GTG TGG C-3', and β -actin reverse: 5'-TCT CTT TAA TGT CAC GCA CGA-3'). Both β -actin and AM targets were amplified in triplicates in the same run using the following cycle scheme: after initial denaturation of the samples at 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s were performed. Fluorescence was measured in every cycle and AM mRNA levels were normalized by the β -actin values

in all samples. A melting curve was run after PCR by increasing the temperature from 50 °C to 96 °C (0.5 °C increments). A single peak was obtained for either β -actin or AM, thus confirming the specificity of the amplification.

Western blotting

PC-12 cells were exposed to hypoxia or normoxia for 24 h in serum-free medium (RPMI 1640 containing 10 µg/ml transferrin, 10 µg/ml insulin, and 50 nM sodium selenite). The supernatants were freeze-dried and resuspended in sample buffer containing β -mercaptoethanol (Novex, San Diego, CA, USA) and the equivalent of 0.5 ml medium was loaded per well. The cell pellets were sonicated in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM sodium orthovanadate, and 1% sodium dodecyl sulfate) and cleared by ultracentrifugation. Protein contents were calculated by the bicinchoninic acid method (Pierce, Rockford, IL, USA) and 25 µg total protein was loaded per well. Synthetic AM (3 ng) was loaded in a separate well as a positive control. Samples were electrophoretically separated on NuPage 12% Bis-Tris gels (Novex), and run at 180 V until the front dye reached the bottom. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a 0.45 µm nitrocellulose membrane at 30 V for 1.5 h. The membrane was blocked overnight with 5% non-fat dry milk in phosphate-buffered saline, and incubated for 1 h in the primary antibody against AM (1:2500). The membrane was further exposed to peroxidase-bound goat anti-rabbit immunoglobulins (Sigma, St Louis, MO, USA), 1:10 000 for another hour, and then peroxidase activity was revealed with the ECL+Plus chemiluminescence kit (Amersham, Arlington Heights, IL, USA) following the manufacturer's instructions. These experiments were repeated three times.

Statistical analysis

The averaged media DA release elicited by the two carotid bodies belonging to the same animal was used. The number of animals used for each time-point or treatment was six to eight. Differences between groups were evaluated by one-way analysis of variance followed by Duncan's *post hoc* test. When only two groups of data were statistically compared, Student's *t*-test was used. *P* values lower than 0.05 were considered statistically significant.

Results

Immunohistochemical techniques clearly demonstrated that AM immunoreactivity is present in the carotid bodies, presenting a granular pattern. Using anti-tyrosine-hydroxylase as a marker of glomus cells, we found a complete colocalization with AM by confocal microscopy

Figure 1 Double immunofluorescence followed by confocal microscopy of a frozen section of a rat carotid body. Tyrosine hydroxylase was demonstrated in red (top left), AM in green (bottom left), and the DNA marker DAPI in blue (top right). The combination of the previous images (bottom right) shows a clear colocalization of tyrosine hydroxylase and AM in the cytoplasm of the glomus cells. Bar=10 μ m.

(Fig. 1), demonstrating that the glomus cells are the source of the immunoreactive AM in the carotid bodies.

To investigate whether AM influences carotid body function, we incubated freshly isolated rat carotid bodies in the presence of 10^{-6} M AM and measured their DA contents at different times. Basal DA release from carotid bodies was 223.6 ± 27.1 pg/20 μ l. Addition of 100 mM KCl, a potent secretagogue, evoked a significant 105.2% increase in DA release. By 5 min after AM treatment we had already found a non-statistically significant trend in DA increase, with maximum levels obtained after 30 min. At this time-point, a 94.2% elevation over basal levels was observed. By 60 min, DA release from the carotid bodies began to decrease. The release of DA at 30 min after AM treatment was indistinguishable from the values elicited by 5 min exposure to KCl (Fig. 2).

A dose-dependent response to AM was observed when the carotid bodies were treated for 30 min with different concentrations of AM (Fig. 3). Concentrations higher than 10^{-6} M AM produced statistically significant increases in DA secretion.

In the *in vivo* study, DA release from the carotid bodies in control animals was 206.1 ± 36.0 pg/20 μ l. This value was similar to the release from carotid bodies of rats killed 90 min after saline injection (216.4 ± 35.4 pg/20 μ l) and these two groups were combined as a single control group. Injection of AM *in vivo* produced an apparent trend towards an increase in DA, although there were no statistically significant differences, with values 263.2 ± 45.5 , 272.3 ± 40.2 , and 263.1 ± 51.6 pg/20 μ l for 30, 60, and 90 min after injection respectively.

Figure 2 DA release, expressed as pg/20 µl, in freshly isolated carotid bodies exposed to 10^{-6} M AM for the indicated times. KCl was added for 5 min as a positive control. Each bar represents the mean and standard deviation of six to eight animals. The asterisks indicate statistical differences from the basal levels ($P < 0.05$).

The cell line PC-12 was used as a model to investigate whether hypoxia induces AM expression and secretion in oxygen-sensitive cells. PC-12 cells cultured under

Figure 3 DA release (expressed as pg/20 µl) from freshly isolated carotid bodies exposed to increasing concentrations of AM for 30 min. Each bar represents the mean and standard deviation of six to eight animals. The asterisks indicate statistical differences with the basal levels ($P < 0.05$).

normoxic conditions showed a slight tendency to decrease their AM expression levels over time, as shown by Northern blot analysis (Fig. 4A). On the other hand, when these cells were exposed to hypoxia, a marked elevation of AM mRNA was observed by 4 h, this up-regulation reaching a maximum at 12 h. After 24 h of hypoxia, the levels of AM mRNA were still much higher than the levels seen in the normoxic cells (Fig. 4).

When the levels of mRNA were quantified by real-time PCR, the trends observed by Northern blotting were confirmed, with statistically higher AM levels seen from 4 h exposure to hypoxia onwards (Fig. 4B). When including treatment (normoxia vs hypoxia) and time (0–24 h) as factors in an analysis of variance, both factors showed statistically significant differences (df 2, $F = 82.352$, $P < 0.001$ for treatments and df 4, $F = 26.985$, $P < 0.001$ for time). Less significant difference *post hoc* analysis showed significant differences between treatments at 4, 8, and 24 h ($P < 0.001$).

Western blot analysis showed that after 24 h exposure to hypoxia, the intracellular contents of AM decreased slightly whereas the amount of AM secreted into the medium increased considerably, suggesting that hypoxia not only increases AM expression but also its release from the secretory granules (Fig. 4C).

Discussion

The present data demonstrate that AM immunoreactivity is expressed by the glomus cells of the carotid bodies. In addition, AM was shown to modulate carotid body function by inducing DA release *in vitro* in a time- and dose-dependent manner. Furthermore, we have shown that AM expression and secretion are elevated upon hypoxic exposure in oxygen-sensitive cells. These observations suggest that AM may act as an autocrine regulator of carotid body physiology. The concentration of AM needed to elicit a statistically significant induction of DA release in our experimental model was 10^{-6} M. This is also the concentration needed for inducing effects on PC-12 signal transduction pathways through the AM gene-related peptide, proadrenomedullin N-terminal 20 peptide (Takekoshi *et al.* 1999). These high concentrations may well be accomplished through an autocrine mechanism in the vicinity of the degranulation sites.

In the time-course experiment, statistically significant difference from the basal levels was achieved after 30 min of incubation, indicating that AM requires the activation of an intracellular signal transduction pathway rather than acting directly on the membrane potential, as does KCl which induces a similar response in only 5 min. In addition, the statistically significant difference was lost by 60 min, suggesting that AM may be degraded by components of the carotid body. A rapid degradation of AM has been reported when exposed to cell membranes from different origins (Lewis *et al.* 1997).

Figure 4 Effects of hypoxia on the expression and secretion of AM from PC-12 cells. (A) Northern blot analysis of PC-12 cells cultured under normoxia or hypoxia for the indicated times. Total RNA (15 µg) was loaded per lane and hybridized with a rat-specific AM probe (upper panel). Equal loading was monitored by ethidium bromide staining of 18 S rRNA (lower panel). (B) The same RNAs were quantified by real-time PCR and corrected by the β-actin values found in the samples. Statistically significant differences were obtained between treatments in the whole experiment (normoxia vs hypoxia, $P<0.001$) and at particular time-points, as indicated by asterisks ($P<0.001$). (C) Western blot detecting the levels of immunoreactive AM in cell extracts (25 µg total protein/lane) and conditioned media (the equivalent of 0.5 ml serum-free medium) of PC-12 cells grown in normoxia or hypoxia for 24 h. Synthetic AM was added (left lane) as a positive control. Unconditioned medium (Uncond.) was added as a negative control.

In the Western blot experiment we found a decrease in the intracellular immunoreactivity accompanied by an increase in the amount of secreted AM. This observation suggests that hypoxia not only increases AM expression, as previously reported in other systems (Garayoa *et al.* 2000, Ogita *et al.* 2001), but it also induces release of the peptide stored in the secretory granules.

The *in vivo* injection of 1 µl 60 nM AM/g BW did not produce statistically significant differences in the amount of DA liberated from the carotid bodies. This concentration was chosen because it has been previously shown to elicit biological effects in rats (Martínez *et al.* 1996). The lack of effect may reflect either the need for higher concentrations in the proximity of the carotid bodies or sequestration of the peptide by the AM-binding protein, complement factor H (Pío *et al.* 2001).

Oxygen sensing is performed by electrically excitable cells located in chemoreceptor organs. These cells are capable of sensing global O₂ tension and produce cardio-respiratory adjustments during hypoxemia. Organs with this ability include carotid and aortic bodies, neuro-epithelial bodies in the lung, and potentially adrenal chromaffin cells in the neonate (López-Barneo *et al.* 2001). The acute response to hypoxia is mediated by oxygen-sensitive ion channels located in the membrane of the glomus cell. Low oxygen tension induces closure of K⁺ channels which results in membrane depolarization, Ca²⁺ influx, transmitter release to the extracellular milieu, and activation of neighbor afferent sensory fibers (López-Barneo *et al.* 2001). On the other hand, intermittent or prolonged exposures to hypoxia produce changes in gene expression and tissue remodeling.

Since there are no available cell lines derived from carotid bodies, many studies have employed cells of the adrenal medulla as models, given their common embryological origin from the neural crest and their demonstrated oxygen-sensing capability (Millhorn *et al.* 1996). Our Northern blot and real-time PCR analysis in PC-12 cells showed that exposure to hypoxia induces overexpression of AM mRNA, with a concomitant increase in the amount of secreted peptide as shown by Western blot analysis of serum-free conditioned medium. Previous studies have shown that addition of extrinsic AM to chromaffin cells stimulates catecholamine release (Kobayashi *et al.* 2001). Thus, we could hypothesize that a similar mechanism may be at work in the carotid body, with hypoxemia inducing overexpression and secretion of AM, which in turn may collaborate in the release of DA from glomus cells.

Unlike its effects in other organs, hypoxia causes the carotid body to grow by increasing the number, size, and excitability of glomus cells (Stea *et al.* 1992). These changes are most likely mediated by the transcription factor, HIF-1 (López-Barneo *et al.* 2001). We have previously demonstrated that AM is transactivated by HIF-1 under hypoxic conditions (Garayoa *et al.* 2000) and

that AM is a growth factor for a number of cells of different tissue origins (Miller *et al.* 1996, Martínez *et al.* 1997). AM has been also characterized as an antiapoptotic factor (Kato *et al.* 1997, Martínez *et al.* 2002). These observations, together with the data presented in this study, indicate that AM may be one of the main intermediaries linking hypoxic stimulus and growth of the carotid body.

An emerging promising therapy for Parkinson's disease is the autotransplant of carotid bodies into the striatum (Espejo *et al.* 1998, Luquin *et al.* 1999). The initial hypothesis was to use the cells from the carotid body as sources of DA for the nigro-striatal circuit, which is DA depleted in Parkinson's patients. Nevertheless, careful analysis of the animals receiving the transplants has shown that the newly formed dopaminergic fibers do not arise from the glomus cells but from the substantia nigra. Apparently, the glomus cells secrete a growth factor that induces recovery of the original neural pattern (Toledo-Aral *et al.* 2002). Hypothetically, AM could be a good candidate for this function given its abundant presence in the carotid body and its characteristics as a potent growth factor (Miller *et al.* 1996, Martínez *et al.* 1997). Investigation in this direction merits further attention.

Numerous studies have shown the involvement of AM in regulating blood pressure through both a direct influence in the blood vessels driving vasodilatation (Parkes 1995, Eto *et al.* 1999, Terata *et al.* 2000) and in an indirect fashion mediated by the central nervous system, which results in vasoconstriction (Mark *et al.* 1997, Saita *et al.* 1998). Here we show a potentially new indirect mechanism of regulating blood flow by eliciting DA release from the carotid bodies. Since DA is a vascular tone modulator (Furukawa *et al.* 2002), this may constitute a new layer of regulation of blood pressure through AM.

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