Tissue hypoxygenation activates the adrenomedullin system in vivo

KARL-HEINZ HOFBAUER,1 BOYE L. JENSEN,2 ARMIN KURTZ,1 AND PETER SANDNER1

1Institut für Physiologie der Universität Regensburg, D-93040 Regensburg, Germany; and 2Institut for Medicinsk Biologi, Department of Physiology and Pharmacology, University of Odense, DK-5000 Odense C, Denmark

Hofbauer, Karl-Heinz, Boye L. Jensen, Armin Kurtz, and Peter Sandner. Tissue hypoxygenation activates the adrenomedullin system in vivo. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R513–R519, 2000.—Our study aimed to investigate the influence of tissue hypoxygenation on the adrenomedullin (ADM) system in vivo. For this purpose, male Sprague-Dawley rats were exposed to normobaric hypoxia (8% oxygen) or to functional anemia [0.1% carbon monoxide (CO)] or to cobalt chloride (60 mg/kg) for 6 h. Messenger RNA levels for ADM and its receptor (ADM-R) were assessed in diverse organs by RNase protection assay. Additionally, ADM protein concentrations in these organs, as in plasma, were determined by a RIA. We found that ADM mRNA abundance increased in response to hypoxia and to CO inhalation up to 15-fold in all organs examined. Similarly, ADM-R mRNA abundance increased during hypoxia and CO inhalation in all organs examined with exception of the liver. The effects of hypoxia and of CO inhalation on ADM and ADM-R mRNAs were mimicked by injection of cobaltous chloride. Hypoxia also significantly increased ADM protein content in all organs, and plasma levels of ADM rose twofold in response to hypoxia and CO inhalation. These findings indicate that tissue hypoxia leads to a widespread activation of the ADM system, which comprises a parallel stimulation of ADM and ADM receptor mRNA as enhanced ADM protein synthesis and secretion. The ADM system may, therefore, play a significant role in the physiological response to tissue hypoxia. It appears that ADM and ADM-R belong to the family of classic oxygen-regulated genes, which are activated by a decrease of the pericellular oxygen tension through the same intracellular signaling cascade.

hypoxia; oxygen; erythropoietin; carbon monoxide; cobaltous chloride; receptor

THE 52-AMINO ACID PEPTIDE adrenomedullin (ADM) was originally discovered as a hypotensive factor produced by human adrenal pheochromocytoma cells (15). Accumulated evidence shows that ADM has a widespread distribution to diverse organ systems and, moreover, that smooth muscle and endothelial cells of the vasculature are major sites of ADM synthesis and release (26). ADM has several other physiological effects apart from vasodilatation. Thus ADM has been reported to cause natriuresis and diuresis (8, 13, 16, 29), to inhibit angiotensin II-induced aldosterone production (1), and to stimulate proliferation of tumor cell lines (17, 18). ADM exerts its effects via specific target cell surface receptors (ADM-R) but also via receptors for calcitonin gene-related peptide, to which ADM is structurally related (9, 14, 28). Despite the wide tissue distribution of ADM and its broad biological activity, a clear physiological role for ADM in control of organ function has not been established. A major reason for this deficit results from the lack of knowledge about the regulation of ADM production and release at the systemic level. There are studies reporting a moderate elevation of ADM plasma levels in a variety of diseases, including cardiovascular, respiratory, hepatic, and renal disorders (5). Thus it has been found that pressure overload and cardiomyopathy increase ADM expression in the ventricles of the heart (22).

More recently, it was reported that hypoxia increases ADM mRNA levels in a human colorectal carcinoma cell line (19) and also in primary cultures of rat ventricular myocytes (7), suggesting that ADM gene transcription could be regulated by the tissue oxygen tension. These findings prompted us therefore to assess the physiological impact of tissue hypoxia for the ADM system in vivo. Because the activity of the ADM system is determined by the production of ADM and by the availability of ADM-Rs as well, we aimed to examine the influence of tissue hypoxia on both ADM and ADM-R gene expression. Furthermore, we intended to rule out if ADM mRNA alterations were paralleled by ADM protein synthesis and/or changes in circulating ADM.

For this purpose, we exposed rats to either normobaric hypoxia; to carbon monoxide, which produces a normocytic anemia by reducing the oxygen carrying capacity of the blood; or injected cobalt chloride, which is already known to activate hypoxia-inducible genes (10). We found that both hypoxia and functional anemia upregulated ADM and ADM-R mRNAs in a variety of organs. Moreover, hypoxic stimulation significantly increased ADM protein levels in these organs as in plasma.

MATERIALS AND METHODS

In vivo protocols. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German Law on the Protection of Animals. Male Sprague-Dawley rats (200–250 g) that had free access to food and water were used for the experiments and treated in the following way: 1) in the control group, animals received no treatment (n = 8); 2) in the...
hypoxia group, the animals were placed in a gas-tight box that was continuously supplied with a gas mixture of 8% O2-92% N2 for 6 h (n = 6); 3) in the carbon monoxide group, the animals were placed in a gas-tight box that was continuously supplied with room air plus 0.1% carbon monoxide (CO) for 6 h (n = 6); and 4) for cobalt treatment, the rats were subcutaneously injected with cobalt chloride (60 mg/kg), and the animals were killed 6 h later (n = 8).

At the end of the experiments, the animals were killed by decapitation and trunk blood was collected for determination of hematocrit and plasma ADM levels. Brains, hearts, kidneys, livers, and lungs were quickly removed, weighed, and rapidly frozen in liquid nitrogen. All organs were stored at −80°C until isolation of total RNA.

Isolation of RNA. Total RNA was extracted from the frozen tissues according to the protocol of Chomczynski and Sacchi (6). In brief, after homogenization in solution D [guanidine thiocyanate (4 M) containing 0.5% N-laurylsarcosinate, 10 mM EDTA, 25 mM sodium citrate, 700 mM β-mercaptoethanol, 2 mM sodium acetate (pH 4), phenol (water saturated), and chloroform] were sequentially added to the homogenate. After cooling on ice and centrifugation at 4°C (10,000 g), the supernatant was precipitated with an equal volume of isopropanol at −20°C. After resuspending the pellet in solution D and precipitating with isopropanol, the resulting RNA pellets were finally dissolved in diethylpyrocarbonate-treated water, the yield of RNA was measured at 260 nm, and the RNA was stored at −80°C until further processing.

RNase protection assay of specific mRNAs. ADM and ADM-R mRNA levels were measured by RNase protection assay as described previously (12). To validate the effect of the protocols, we included measurements of mRNA for the classical oxygen-sensitive gene product, the hormone erythropoietin (EPO), as previously described (21). Finally, an intraperitoneal injection of cobalt chloride led to three- to fourfold increases of ADM mRNA, however, markedly less effective than hypoxia or cobalt. Similarly, ADM-R mRNA relative to normoxic controls, the hybridization signals obtained for ADM and ADM-R mRNAs were standardized with respect to the amount of total RNA used for the respective RNase protection assays. This comparison reveals that lung, kidney, and heart of normoxic animals display a substantial basal expression of the ADM gene, whereas ADM mRNA is less abundant in brain and liver (Fig. 1A). The ADM-R gene is predominantly expressed in the lung but also in the liver and kidneys of normoxic animals, whereas brain and heart only show little expression of this gene (Fig. 1B). Figure 1C shows the abundance of mRNA for the housekeeping gene β-actin in the different organs of normoxic rats. It can be seen that lung, kidney, and brain show higher abundance of β-actin mRNA than liver and heart.

To induce or to mimic tissue hypoxia, we used three well-established maneuvers, namely normobaric hypoxia with an inspiratory oxygen fraction of 8% for 6 h (n = 6), inhalation of 0.1% CO for 6 h (n = 6), and, finally, an intraperitoneal injection of cobalt chloride (60 mg/kg), which was allowed to work for 6 h (n = 8). Compared with normoxic controls, the hypoxic protocols applied in this study had no effect on mRNA levels for β-actin in any organ studied, which is shown for kidneys by a representative autoradiogram (Fig. 2, bottom). Therefore, β-actin was used for internal standardization of the hybridization products for ADM and ADM-R mRNAs.

Figure 2, top and middle, shows an autoradiogram of a RNase protection assay for ADM and ADM-R mRNA after application of the different experimental protocols for 6 h. In this experiment, 20 µg of rat kidney total RNA was used for hybridization. In the kidney, hypoxia and CO induced 2.5- and 3.5-fold increases of ADM mRNA, respectively, whereas cobalt had no significant effect (Figs. 2, top, and 3A). Also ADM-R mRNA in the kidney was significantly increased by hypoxia and by CO (Figs. 2, middle, and 3B). The effect of cobalt on kidney ADM-R mRNA did not reach a level of significance on the basis of eight animals.

Figure 4 shows the effect of the different types of hypoxic stimulation on ADM and ADM-R mRNA abundance in rat lung. Normobaric hypoxia and cobaltous chloride led to three- to fourfold increases of ADM mRNA relative to normoxia. CO inhalation also significantly increased ADM mRNA, however, markedly less effective than hypoxia or cobalt. Similarly, ADM-R mRNA was measured on the pre-extracted (Sep-Pak C18) plasma, and tissues were sampled by a sensitive RIA employing an antibody against rat ADM (Phoenix Pharmaceuticals). For the RIA, 100 µl of each sample was used.

Statistics. Levels of significance between groups were calculated using the unpaired Student’s-t-test and by ANOVA. P < 0.05 was considered significant.

RESULTS

To compare the basal expression levels of ADM and of ADM-R mRNAs between the different organs of normoxic control rats, the hybridization signals obtained for ADM and ADM-R mRNAs were standardized with regard to the amount of total RNA used for the respective RNase protection assays. This comparison reveals that lung, kidney, and heart of normoxic animals display a substantial basal expression of the ADM gene, whereas ADM mRNA is less abundant in brain and liver (Fig. 1A). The ADM-R gene is predominantly expressed in the lung but also in the liver and kidneys of normoxic animals, whereas brain and heart only show little expression of this gene (Fig. 1B). Figure 1C shows the abundance of mRNA for the housekeeping gene β-actin in the different organs of normoxic rats. It can be seen that lung, kidney, and brain show higher abundance of β-actin mRNA than liver and heart.

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mRNA abundance also increased in the lung two- to fourfold during hypoxia, CO, or cobalt treatment. In the heart, hypoxia and CO were equally effective in producing a fourfold increase of ADM mRNA, whereas cobalt was somewhat less effective in this respect (Fig. 5A). Also, ADM-R mRNA increased up to threefold in the heart under the three hypoxic maneuvers, with cobalt as the most effective stimulus (Fig. 5B).

In the present study marked effects of hypoxia on ADM mRNA was observed in the brain. Thus hypoxia and CO raised ADM mRNA levels 14-fold, and cobalt injections led to sixfold increases (Fig. 6A). ADM-R mRNA in the brain was modestly stimulated by CO, cobalt, and hypoxia (Fig. 6B).

Finally, in the liver, hypoxia, CO, and cobalt produced 6- to 14-fold increases of ADM mRNA, with cobalt being the most effective stimulus (Fig. 7A). In contrast to all other organs examined in this study, ADM-R mRNA did not increase in the liver in response to hypoxia, CO, or cobalt.

Fig. 2. Top and middle autoradiogram of a RNase protection assay for ADM (top) and ADM-R (middle) mRNA showing protected ADM and ADM-R cRNA probes that were hybridized with 20 µg total kidney RNA obtained from rats exposed to normoxia (controls), hypoxia (8% O2), carbon monoxide (0.1% CO), or cobalt chloride (60 mg CoCl2/kg) and 20 µg tRNA (negative control). In last lane at right, intact single stranded ADM and ADM-R cRNA probes are seen. Bottom: autoradiogram of a RNase protection assay for β-actin mRNA showing the protected cRNA probe that was hybridized with 1 µg total kidney RNA, obtained from rats exposed to same conditions as mentioned above.
to the different protocols of hypoxia. In contrast, ADM-R mRNA levels in the liver were significantly decreased by the two different approaches of normobaric hypoxia and by cobalt (Fig. 7B). This effect was not caused by differences in the β-actin level between controls and hypoxic animals, which did not differ between normoxic and hypoxic conditions.

As a functional control of the effectiveness of the protocols to induce tissue hypoxia, we also assayed mRNA levels for the classical oxygen-regulated hormone EPO. In the two major organs of EPO mRNA expression, namely kidney and liver, we noted significant increases of mRNA abundance up to 25-fold above control levels (Fig. 8).

To examine whether the induction of ADM mRNA by hypoxia in various organs was associated with changes of ADM protein in these different organs and with changes in ADM secretion to the circulation, we assayed ADM immunoreactive peptide levels in organs and plasma. Plasma ADM concentration was significantly increased by normobaric hypoxia and by CO, whereas cobalt injections had no significant effect after 6 h (Fig. 9). The upregulation of ADM mRNA in kidneys, lungs, hearts, brains, and livers was paralleled by significant increase in ADM proteins (Table 1), which was highest in lungs and livers (2.5-fold and 2.4-fold, respectively), followed by hearts (1.9-fold), brains (1.8-fold), and kidneys (1.7-fold).

**DISCUSSION**

The present study was undertaken to test the hypothesis that synthesis and release of the vasoactive peptide ADM is regulated by the oxygen content of the blood. Under physiological conditions we found that ADM is abundantly expressed in the lung, kidney, and heart, which is in accordance with several previous reports (1, 12, 17). Moreover, the ADM-R was predominantly expressed by the lungs and at lower levels by the kidney, liver, heart, and brain, which also fits with previous receptor binding and expression studies (14, 20). Thus the ADM system has a nearly ubiquitous distribution in rat tissues.

The main finding of the present study is that tissue hypoxia induced by either arterial hypoxia or a reduction of oxygen transport is a general stimulus for ADM mRNA abundance in all organs tested. Our findings thus may provide a good explanation of the demonstration of elevated ADM mRNA in ischemic regions of the brain (31). The concomitant increase of ADM peptide in all organs and the plasma during hypoxia or anemia supports the inference that the
increase of ADM mRNA has led to an increased synthesis and release of ADM peptide. These data are therefore in good agreement with previous in vitro studies reporting that hypoxia increases ADM mRNA levels and ADM protein synthesis in tumor cells (19). It should be mentioned in this context that in lungs of rats exposed to hypoxia for 7 days no increase of ADM protein or ADM mRNA was measured (33). This lack of change may be due to the more moderate hypoxic conditions (animals were kept at 10% oxygen) used for the chronic experiments but may also indicate that the stimulation of ADM gene expression by hypoxia is temporally transient. From cell culture studies it was suggested that the ADM gene may be regulated similar to classic oxygen-regulated genes, which are under the control of hypoxia-inducible transcription factor-1 (HIF-1; 4, 32). In fact, putative consensus sites for HIF-1 binding have been localized to the 5' promoter region of the ADM gene (7). Our finding that cobalt mimics the effect of hypoxia on ADM gene expression in vivo together with the previous observation that cobalt stimulates ADM gene expression in a carcinoma cell line in vitro (19) supports the conclusion that ADM in fact belongs to the family of oxygen-regulated genes that are transcriptionally activated by a decrease of the pericellular oxygen tension (4). Although all of these genes are strongly activated by hypoxia in vitro, the majority of them respond only modestly, if at all, to hypoxia or anemia in vivo (2, 3, 24). This suggests that they are physiologically controlled by parameters other than the oxygen tension. In contrast, ADM gene expression appears to be importantly regulated by the oxygen tension in vivo. In this context, the acute and marked sensitivity of ADM to hypoxia in vivo mimics that of the hormone EPO, which is considered the prototype of an oxygen-regulated gene product (11).

In addition to the marked effect of hypoxia on ADM mRNA and protein levels, ADM-R mRNA abundance was also highly sensitive to oxygen. With the exception of the liver, ADM-R mRNA was stimulated by hypoxia, anemia, and cobalt in all organs examined. We speculate therefore that the ADM-R gene may likewise belong to the family of oxygen-regulated genes. In line with our data is the previous observation that ADM binding sites in the lung increase during chronic hypoxia (33). At present, we are not able to provide any information about putative HIF-1 consensus sequences in the ADM-R gene. The cause and functional significance of the inverse regulation of ADM-R mRNA in the liver cannot be satisfactorily answered by our data. We assume that the ADM-R gene is negatively controlled.
by yet unknown factors that become particularly active in the liver during hypoxia.

If it is assumed that the abundance of ADM-mRNA and of ADM-R mRNA reflects the local production rates of ADM and of the ADM receptor proteins, respectively, our data would suggest an amplified activation of local ADM systems during tissue hypoxia. Indirect evidence for such a synergism comes from the observation that the vasodilatory effect of ADM in the pulmonary vascular bed is increased during hypoxia (33). A similar concomitant increase of ligand and its receptor in hypoxic tissues has previously been described for vascular endothelial growth factor (VEGF; 25, 27). This upregulation of the VEGF system is thought to improve oxygen delivery to chronically hypoxic tissues primarily by the formation of new capillaries.

Perspectives

Because vasodilation is the best established physiological effect of ADM, we would hypothesize that the upregulation of the ADM system in vivo mediates vasodilatation in hypoxic tissues and in consequence improves blood and oxygen delivery in states of an imbalance between oxygen demand and oxygen delivery. Thus, in addition to the well-known hypoxia-induced responses of erythropoiesis and angiogenesis, the ADM system would provide a third paracrine pathway that directly influences vasoreactivity to match oxygen delivery. The increase of plasma ADM concentration during hypoxia may reflect a spillover effect rather than a regulated secretion into the bloodstream to reach distant target cells. However, we cannot presently rule out that the increase of plasma ADM during hypoxia also has a physiological significance at targets where ADM is not locally produced and released. To establish the significance of the ADM system during hypoxia requires the emergence of specific ADM receptor antagonists or the application of transgenic strategies.

Taken together, our results indicate that tissue hypoxegenation is a powerful physiological stimulus for ADM and ADM-R mRNA expression as ADM protein synthesis in multiple organs in vivo. This suggests that the ADM system could play an important role in the regulation of tissue hemodynamics during conditions of hypoxia.

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ADRENO MEDULLIN AND HYPOXIA


