Induction of Adrenomedullin by Hypoxia in Cultured Retinal Pigment Epithelial Cells

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PURPOSE. To explore the effects of hypoxia on the production and secretion of adrenomedullin (ADM) and endothelin (ET-1) in human retinal pigment epithelial (RPE) cells.

METHODS. RPE cells were cultured under normoxic or hypoxic (1% O₂) conditions. Expression of ADM and ET-1 was examined by Northern blot analysis and radioimmunoassay. Effects of ADM and ET-1 on the number of RPE cells were examined by modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

RESULTS. ADM mRNA expression levels and immunoreactive ADM levels in the medium were increased by hypoxia in all three human RPE cell lines (ARPE-19, D407, and F-0202). Immunoreactive ET was detected in the cultured media of D407 cells and ARPE-19 cells and identified as ET-1 by reversed-phase high performance liquid chromatography. Hypoxia treatment for 48 hours increased immunoreactive ET levels approximately 1.3-fold in the cultured media of D407, but not ARPE-19 cells. Hypoxia decreased the number of ARPE-19 cells and F-0202 cells, and the treatment with ADM ameliorated the hypoxia-induced decrease in the cell number. In contrast, exogenously added ET-1 had no significant effects on the number of ARPE-19 cells under normoxia and hypoxia.

CONCLUSIONS. Hypoxia increased the expression of ADM in all three human RPE cell lines, whereas the induction of ET-1 by hypoxia was found only in D407 cells. ADM induced by hypoxia may have protective roles against hypoxic cell damage in RPE cells. (Invest Ophthalmol Vis Sci. 2001;42:1080–1086)

Adrenomedullin (ADM) is a vasorelaxant peptide originally identified from human chromomycotoma. It has been reported that ADM is produced by various human tissues and cells and has a variety of biologic actions. In eyes, ADM has a relaxant effect on iris sphincter muscle and an ocular hypotensive effect. ADM has positive or negative effects on cell proliferation, depending on the cell types. We have recently reported elevated ADM levels in the vitreous fluid in patients with proliferative vitreoretinopathy. In addition, ADM is secreted from cultured human retinal pigment epithelial (RPE) cells and has proliferative effects on these cells.

Endothelin (ET)-1 is a vasoconstrictor peptide originally isolated from vascular endothelial cells. It is produced in numerous organs, has diverse biologic functions, and is abnormally distributed in the eye. Immunocytochemical studies show patchy immunostaining for ET-1 in RPE cells in rats. ET-1 has various physiological influences in the eye, such as regulating choroidal and retinal circulation, intraocular pressure regulation, and modulatory effects in inflammation. Moreover, ET-1 has a stimulatory effect on the proliferation of various types of cells, including corneal epithelial cells.

Hypoxia induces the gene expression of a variety of proteins, such as erythropoietin, vascular endothelial growth factor, and glucose transporters. On the contrary, expression of pigment-epithelium derived factor (PEDF) decreases under hypoxia. PEDF is a neurotrophic factor initially identified in conditioned media from cultured human fetal RPE cells and acts as a potent inhibitor of angiogenesis.

We and other investigators have reported that hypoxia induces ADM expression in colorectal carcinoma cells, coronary artery endothelial cells, cardiomyocytes, Madin-Darby canine kidney cells, and rat mesangial cells. Some investigators have reported that hypoxia induces ET-1 gene expression in cultured endothelial cells, but others have said that it decreases ET-1 production. Ischemia and hypoxia are involved in the pathophysiology of some ocular diseases, such as ischemic retinopathies.

Materials

Materials

Human ADM: human ADM-(22-52), an ADM receptor antagonist; and ET-1 were obtained from the Peptide Institute (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM), 1:1 mixture of DMEM and nutrient mixture F12, minimum essential medium (MEM), and penicillin-streptomycin were obtained from Life Technologies (Rockville, MD). Fetal bovine serum (FBS) was obtained from CSL (Parkville, Victoria, Australia). Basic fibroblast growth factor (bFGF) was obtained from Pepro Tech EC (London, UK). [α-32P]-dCTP was obtained from Amersham Pharmacia Biotech (Tokyo, Japan) and [125I]-insulin from Daichi Kagaku (Tokyo, Japan). Restriction endonucleases were purchased from Takara (Osaka, Japan) and New England BioLabs (Boston, MA). Cell Counting Kit-8 was purchased from Dojindo (Kumamoto, Japan). A low-temperature O₂/CO₂ incubator (model-9200; Wakenyaku, Kyoto, Japan) was used in hypoxia experiments.

Cell Culture

The human RPE cell lines ARPE-19 and D407 were kindly given by Leonard M. Hjelmeland (Department of Biological Chemistry, University of Washington, Seattle, USA). Cell culture media were prepared from DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a humidified incubator with 5% CO₂ at 37 °C.
The ARPE-19 cell line was derived from a 19-year-old man. The cells from passages 13 through 16, which are not considered to be transformed, were used in the experiments. ARPE-19 cells were cultured in a 1:1 mixture of DMEM and nutrient mixture F12 containing 10% FBS, 2 mM t-glutaminate, and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37°C under 5% CO2 and 95% room air. D407 cells were derived from a 12-year-old white male child. Cells maintained in culture for more than 200 passages were used in the experiments. D407 cells were cultured in DMEM containing 10% FBS, 2 mM t-glutaminate, and antibiotics.

F-0202 human RPE cells were derived from human fetal eyes, as described previously. Cells from passages 7 through 9 were used in the experiments. F-0202 cells were cultured in MEM containing 10% FBS, bFGF (10 ng/ml), and antibiotics.

In hypoxia experiments, human RPE cells were cultured in a chamber with 5% CO2, 94% N2, and 1% O2. The cells were cultivated under normoxia or hypoxia for 12, 24, and 48 hours. They were harvested for RNA extraction, and the cultured media were collected for the measurement of immunoreactive adrenomedullin (IR-ADM) and immunoreactive endothelin (IR-ET). The experiments were performed in five dishes per each treatment.

**Peptide Extraction and Radioimmunoassay**

Peptides in the medium were extracted with a Sep-Pak C18 cartridge (Waters, Milford, MA). IR-ADM in the extract was measured by radioimmunoassay, as previously reported, using the antiserum against human ADM (1-52) (antibody 102). Cross reaction with ADM-glycine (a gift of Kazuo Kitamura, Miyazaki Medical College, Japan) was 40%, but less than 0.001% with calcitonin gene-related peptide (CGRP), ET-1, and other peptides tested. IR-ET was measured by radioimmunoassay using antibody against ET-1 (BP6, a gift from Stephen R. Bloom and Mohammad A. Ghatei, Hammersmith Hospital, London, UK), as previously reported. The ET-1 antibody showed less than 0.001% cross reaction with ADM and other peptides.

Chromatographic characterization of IR-ET in the culture media of ARPE-19 and D407 cells was performed by reversed-phase high performance liquid chromatography (HPLC) using muBondapak C18 column (3.9 x 300 mm; Waters). The extract was reconstituted in 0.1% (vol/vol) trifluoroacetic acid and loaded onto the column. Peptides were eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid from 10% to 60% at a flow rate of 1 ml/min per fraction over 50 minutes. Each fraction (1 ml) was collected, dried by air, and assayed.

**RNA Extraction and Northern Blot Analysis**

Total RNA was extracted from cultured cells by the guanidium thiocyanate-cesium chloride method and subjected to Northern blot analysis, as previously reported. The Northern probe for ADM mRNA was the HindIII/EcoRI fragment of pBSAdM2. The probe for ET-1 mRNA was the BamHu/EcoRI cDNA fragment of pBHET-1 (a gift from Stephen R. Bloom and Philip M. Jones, Hammersmith Hospital). Expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined as an internal control. The probe for G3PDH mRNA was the NcoI/Pst fragment derived from a rat 720-bp G3PDH cDNA fragment (nucleotides 5-104 and 368-987) subcloned into a vector (pGEM-T; Promega, Madison, WI).

Radioactive signals were detected by exposing the filters to x-ray film (X-AR5; Kodak, Rochester, NY) or with an image analyzer (Bioimage Analyzer BAS 1500; Fuji Film, Tokyo, Japan). The intensity of hybridization signals representing ADM mRNA was normalized with that representing G3PDH mRNA.

**Analysis of Apoptosis**

The effect of ADM on apoptosis was examined using an apoptosis screening kit (Wako, Osaka, Japan; the modified TdT-mediated dUTP nick-end labeling [TUNEL] method). ARPE-19 cells were cultured with or without human ADM (10-7 M) for 24 hours under hypoxic conditions. The cells were also cultured under normoxic conditions (normoxia control) or treated by 100 ng/ml actinomycin D (A01D) under normoxia as a positive control for apoptosis. Apoptosis was detected by the apoptosis screening kit, according to the manufacturer’s protocols. The cells were permeabilized and reacted with terminal deoxynucleotidal transferase (TdT) solution. The reaction product representing DNA fragmentation was detected by peroxidase-conjugated antibody. The OD of 490 nm in five wells per each treatment was measured by spectrophotometer.

**Statistical Analysis**

Data are expressed as mean ± SEM, unless otherwise stated. Statistical analysis was performed by one-way analysis of variance followed by the Fisher’s protected least significant difference test.

**RESULTS**

**IR-ADM and IR-ET-1 Levels under Hypoxic Conditions**

To study the regulation of ADM and ET-1 secretion, we cultured ARPE-19, D407 and F-0202 cells under hypoxic conditions (5% CO2, 94% N2, and 1% O2). IR-ADM accumulated time dependently in the media of ARPE-19 cells and D407 cells (Figs. 1A, 1B) but remained constant in F-0202 cells at 24 hours and 48 hours (Fig. 1C), both under normoxia and under hypoxia. IR-ADM levels in the culture media of ARPE-19 cells were increased under hypoxic conditions approximately 1.7-fold at 24 hours (P < 0.0005) and approximately 1.5-fold at 48 hours (P < 0.0001), compared with normoxic control (Fig. 1A). IR-ADM levels in the culture media of D407 cells were increased under hypoxia approximately 2.7-fold at 24 hours (P < 0.0005) and approximately 4.6-fold at 48 hours (P < 0.0001; Fig. 1B). IR-ADM levels in the culture media of F-0202 cells were increased under hypoxia approximately 1.4-fold at 24 hours (P < 0.01) and at 48 hours (P < 0.05; Fig. 1C).
IR-ET levels in the culture media of ARPE-19 cells and D407 cells under normoxia were 2.05 ± 0.14 and 16.36 ± 0.87 femtomoles/105 cells per 24 hours, respectively (Figs. 2A, 2B). In contrast, IR-ET was not detectable in the culture media of F-0202 cells (1.5 femtomoles/105 cells per 24 hours). IR-ET levels in the culture media of D407 cells were increased under hypoxic conditions approximately 1.3-fold (P < 0.001) at 48 hours (Fig. 2B). There were no significant changes in IR-ET concentrations in the culture media of ARPE-19 cells between normoxia and hypoxia both at 24 hours and 48 hours (P > 0.2; Fig. 2A).

HPLC Analysis of IR-ET

The identity of IR-ET in the media of ARPE-19 and D407 cells was assessed by reversed-phase HPLC. Reversed-phase HPLC of the culture media extracts of ARPE-19 and D407 cells showed...
a peak in the position of ET-1, indicating that the IR-ET in the media was ET-1 (Figs. 3A, 3B).

ADM mRNA and ET-1 mRNA in the RPE Cells during Hypoxia

Northern blot analysis showed that hypoxia induced expression of ADM mRNA in all three RPE cell lines (Fig. 4). The expression levels of ADM mRNA were increased 1.2, 1.4, and 1.4-fold in ARPE-19 cells, and 4.0, 5.1, and 7.5-fold in D407 cells at 12, 24, and 48 hours, compared with normoxic control, respectively (Figs. 4A, 4B). By contrast, there was only a small increase (approximately 1.2-fold) in the ADM mRNA expression levels by 48 hours of hypoxia and no change by 12 hours and 24 hours of hypoxia in F-0202 cells (Fig. 4C).

ET-1 mRNA was not detectable in all three human RPE cell lines (ARPE-19, D407, and F-0202) both under normoxic and hypoxia according to Northern blot analysis. Northern blot analysis detected ET-1 mRNA in D407 cells treated with interferon-γ (100 U/ml) or interleukin-1β (1 ng/ml) for 24 hours (data not shown), indicating that the magnitude of the ET-1 mRNA expression in normoxic or hypoxic conditions was not so large as that induced by these cytokines. ET-1 mRNA was detectable in ARPE-19, D407, and F-0202 cells cultured under normoxia and hypoxia by reverse transcriptase-polymerase chain reaction (RT-PCR; data not shown).

Effects of ADM and ET-1 on the Number of Human RPE Cells

The effects of ADM and ET-1 on the number of RPE cells were analyzed by the modified MTT assay. Exogenously added human ADM ($10^{-7}$ M) increased the cell number under normoxic conditions both in ARPE-19 cells and F-0202 cells (Fig. 5), as previously reported. Hypoxia treatment for 24 hours significantly decreased the number of RPE cells (Fig. 5). Exogenously added ADM ($10^{-7}$ M) ameliorated the hypoxia-mediated decrease in the cell number, but lower concentrations of ADM ($10^{-9}$ and $10^{-8}$ M) had no significant effects on the cell number under hypoxia. In contrast, exogenously added ET-1 ($10^{-9}$–$10^{-7}$ M) had no significant effects on the cell number of ARPE-19 cells under both normoxic and hypoxic conditions (data not shown).

Treatment with $10^{-7}$ M ADM (22-52) further decreased the cell number under hypoxic conditions in both ARPE-19 and F-0202 cells. Treatment with an anti-ADM antibody also decreased it. These observations suggest that the IR-ADM secreted by ARPE-19 cells or F-0202 cells stimulated the cell growth or protected the cell damage under hypoxic conditions.

Analysis of Apoptosis

Significant increases in the index of apoptosis were observed in the ARPE-19 cells cultured under hypoxic conditions or

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**Figure 3.** Reversed-phase HPLC of IR-ET in the culture medium extracts of human RPE cells (A: ARPE-19; B: D407). Arrows 1, 2, and 3: Positions of ET-1, ET-2, and ET-3, respectively. Dotted lines: Gradient of acetonitrile.

**Figure 4.** Northern blot analysis of ADM mRNA in three lines of human RPE cells (A: ARPE-19; B: D407; C: F-0202) under normoxic (N) or hypoxic (H) conditions for the time indicated. Each lane contains 15 μg total RNA. G3PDH mRNA served as an internal control. Data are from one of three independent experiments with similar results.
treated with ActD (100 ng/ml; Fig. 6). Exogenously added human ADM (10^{-7} M) had no significant effects on the increased index of apoptosis under hypoxic conditions, suggesting that the effects of ADM on the cell number of RPE cells under hypoxic conditions are not mediated by its antiapoptotic actions.

**DISCUSSION**

The present study has shown that hypoxia induced the expression of ADM in human RPE cells and that exogenously added ADM abolished the hypoxia-induced decrease in the number of the RPE cells. Treatment with an ADM antagonist or an ADM antibody decreased the number of ARPE-19 cells and F-0202 cells. These observations raised the possibility that ADM induced by hypoxia may have protective roles against cell damage caused by hypoxia in RPE cells. In addition, the secretion of ET-1 was observed in two of three RPE cell lines. A small increase in the ET-1 secretion by hypoxia was found in D407 cells. The negative results in the Northern blot analysis of ET-1 mRNA in ARPE-19 and D407 cells may have been due to the treatment with ActD (100 ng/ml; Fig. 6). Exogenously added human ADM (10^{-7} M) had no significant effects on the increased index of apoptosis under hypoxic conditions, suggesting that the effects of ADM on the cell number of RPE cells under hypoxic conditions are not mediated by its antiapoptotic actions.

**Figure 6.** Apoptosis under hypoxic conditions and effects of ADM on apoptosis in ARPE-19 cells. The cells were cultured with or without ADM (10^{-7} M) for 24 hours under hypoxic conditions and also with or without 100 ng/ml ActD under normoxia. Apoptosis in five wells per each treatment was detected by an apoptosis screening kit. Data are presented as the ratio of the index of apoptosis in the cells with each treatment to the mean value of normoxic controls (the mean of normoxic control 5 = 100; mean ± SEM, n = 5). **## P < 0.001; NS, not significant. N, normoxia; H, hypoxia. Data shown are from one of three independent experiments with similar results.**
lower sensitivity of the method, because ET-1 mRNA was detectable in both cells by RT-PCR.

These findings are consistent with previous reports on ADM induction by hypoxia in cultured tumor cells, and in cultured normal cells derived from cardiovascular and renal tissues. However, there was some difference in the magnitude of the ADM induction by hypoxia among three RPE cell lines (ARPE-19, D407, and F-0202) examined, which may reflect the variability in their metabolic properties. D407 cells were the most sensitive to hypoxia in the induction of ADM and ET-1. It is also noteworthy that IR-ADM accumulated time dependently in the media of ARPE-19 and D407 cells but remained constant in F-0202 cells. The amount of the IR-ADM secreted by F-0202 cells in the first 24 hours of culture was similar to that in the next 24 hours under normoxic or hypoxic conditions (data not shown). ADM secreted by F-0202 cells may bind to the ADM receptors on the F-0202 cells and be degraded more rapidly during the culture than the other two cell lines. It is known that ADM-binding sites are expressed in various kinds of tissues and cells.

Exogenously added ADM abolished the hypoxia-induced decrease in the cell number of ARPE-19 and F-0202 cells, only at the highest concentration (10−7 M). In addition, studies using an ADM receptor antagonist and an anti-ADM antibody showed that endogenously produced ADM is necessary for the proliferation or the survival of the RPE cells under hypoxia. It was reported that ADM suppressed apoptosis induced by serum deprivation in cultured endothelial cells. In this context, we observed that apoptosis occurred in ARPE-19 cells under 24-hour hypoxia but was not affected by the treatment of 10−7 M ADM. It is therefore plausible that the effects of ADM on the cell number of RPE cells under hypoxic conditions are independent of its antiapoptotic actions. Further studies are required to clarify whether the ADM action on the cell number under hypoxic conditions was mediated by its growth stimulatory effects or certain protective effects working in a different manner from antiapoptosis.

By contrast, ET-1 had no significant effects on the number of ARPE-19 cells under normoxia and hypoxia. There have been many reports that showed stimulatory effects of ET-1 on the cell growth in various kinds of cells, and ET-1 has been supposed to be a mitogenic peptide. Our findings suggest that ADM rather than ET-1 is involved in the growth or survival of RPE cells. Another possible role of ADM induced by hypoxia may be vasodilatation. Whereas ET-1 is a potent vasoconstrictor peptide, ADM has a vasodilator action in various types of vascular tissues including retinal arteries. ADM induced by hypoxia in the RPE cells may ameliorate ischemia in the eyes through its vasodilator action.

Ischemia and hypoxia are involved in the pathophysiology of some ocular diseases, such as ischemic retinopathies. The RPE cells are located close to the choroidal capillaries, however, and are less likely to be affected in an ischemic condition than the overlying neuronal retina. ADM is expressed in various types of cells, including neurons and astrocytes. It is therefore plausible that ADM production is also increased in the neuronal retina under the ischemic conditions.

Another implication of the present study may be physiological roles of ADM in embryonic development of the RPE cells. Low oxygen tension (≤5% O2) is known to play a critical role in embryonic development. It was reported that placental PO2 levels were low (17.9 ± 6.9 mm Hg, mean ± SD) between 8 and 10 weeks' gestation but were increased to 60.7 ± 8.5 mm Hg at 12 to 13 weeks, which suggests that the embryo is under hypoxic conditions, similar to the hypoxic culture conditions (1% O2) used in this study. Hypoxic conditions during the embryonic development may stimulate the RPE cells to secrete ADM, which may promote the growth of the cells as an autocrine or paracrine factor.

In this study, we have shown that hypoxia induces ADM expression in human RPE cells. Exogenously added ADM abolished the hypoxia-induced decrease in the cell number of the RPE cells. These findings suggest that ADM induced by hypoxia has protective roles against hypoxic cell damage in RPE cells.

References


