Role of Oxygen on Growth Rate and Gene Activity in Cultured Chick-Embryo Heart Cells

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Environmental oxygen is shown to have a regulatory role in growth rate and the mechanism of gene de-repression in chick-embryo heart cells; it modulates the intracellular concentration of polyamines, and this appears to be closely related to histone acetylation.

Many studies on the effect of different oxygen tensions in tissues (Cryer & Bartley, 1973) and in animal-cell cultures (Karsten *et al.*, 1973) have been made to define the role of this essential element in cell metabolism. These studies are of particular interest in understanding the biochemical events that occur during the pathological states of hypoxia or hyperoxia. The shift from an aerobic to an anaerobic environment has been extensively studied in relation to carbohydrate metabolism (Kilburn *et al.*, 1969; Kittlick & Neupert, 1975), whereas little is known about the regulatory role of oxygen in nucleic acid biosynthesis (Rueckert & Mueller, 1960; Hollenberg, 1971) and, in particular, at what level it acts primarily. To gain some information about the possible role of oxygen at the transcriptional level, we have studied the effect of oxygen on nucleic acid synthesis in relation to the behaviour of some aliphatic amines such as spermine and spermidine. Studies on these biogenic amines suggest that they are related to the regulatory mechanism of nucleic acid synthesis and the cellular proliferation rate (see reviews by Cohen, 1971; Bachrach, 1973). Further, in the light of our finding (Moruzzi *et al.*, 1974) that a relationship exists between polyamines and the chemical modification of histones, we have studied the acetylation of histone fractions, which may play a major role in the gene de-repression mechanism (Allfrey *et al.*, 1963; Pogo *et al.*, 1968).

Materials and Methods

The biological model chosen for the present study were single beating-heart cells obtained from ventricles of 8–10-day-old chick embryos by repeated trypsin treatment as described by DeHaan (1967). Cells were plated in culture dishes (Falcon) at a density of 3×10^6 cells/60 mm plate in Eagle's minimum essential medium with Earle's salts (Grand Island Biological Co., Grand Island, N.Y., U.S.A.) buffered with 25 mM-Hepes [2-(N-2-hydroxyethyl-

piperazin-N'-yl)ethanesulphonic acid; Eurobio Laboratories, Paris, France], pH 7.4, and supplemented with 10% (v/v) inactivated calf serum (Eurobio). Plates were incubated at 37°C in suitable sealed chambers with the desired oxygen tension. Gas mixtures, prepared with the aid of calibrated flowmeters (Brooks Instrument Co., Hatfield, Pa., U.S.A.), consisted of N₂+O₂ (20:80, 60:40, 80:20, 90:10 and 95:5). The gas mixture was renewed daily. About 80% of the cells were beating-muscle cells. The cultures were tested at 24 h intervals during the 3-day growth period studied for DNA, RNA, polyamines and histone acetylation. To determine the rate of [*Me*-³H]thymidine incorporation into DNA, the cultures were overlaid with 2.5 μCi of [*Me*-³H]thymidine (5.0 Ci/mmol; The Radio-chemical Centre, Amersham, Bucks., U.K.)/ml and incubated for 2 h before harvesting. The rate of [³H]uridine incorporation into RNA was determined after addition of 5 μCi of radioisotope (26.2 Ci/mmol; New England Nuclear Co., Boston, Mass., U.S.A.)/ml to similar cultures 1 h before harvest. Exposure to [*Me*-³H]thymidine or [³H]uridine was stopped by decanting the medium and washing the monolayers three times with ice-cold physiological saline solution. Eight culture plates (for each assay) were scraped with a silicone-rubber spatula and the collected material was pooled. The cells, suspended in physiological saline solution, were precipitated twice with HClO₄ to a final concentration of 3% (v/v) and centrifuged at 800g for 10 min. The supernatant solution was used for polyamine determination and the pellet was dissolved in 0.1 M-NaOH at 37°C overnight. Portions from each hydrolysate were removed for measurement of DNA and RNA radioactivity in a Packard Tri-Carb spectrometer by using 10 ml of scintillation 'cocktail' (Insta Gel; Packard, La Grange, Ill., U.S.A.). Portions were used for determination of protein content (Lowry *et al.*, 1951). The acidic extract was analysed for polyamines by t.l.c. of their dansyl derivatives on silica-gel plates

with ethyl acetate/cyclohexane (2:3, v/v) as solvent, by the method of Seiler & Wiechmann (1970). The relative intensity of fluorescence of the polyamine derivatives was assayed by scanning the plates in a Turner model 111 fluorimeter equipped with a recorder.

To measure the rate of [$1\text{-}^{14}\text{C}$]acetate incorporation into histone fraction, the cells were exposed to $1\mu\text{Ci}$ of sodium [$1\text{-}^{14}\text{C}$]acetate (56mCi/mmol ; The Radiochemical Centre)/ml for the final 1 h of culture. Incorporation was stopped as described above and the cells (from eight plates) were harvested by scraping them into phosphate-buffered saline and centrifuging at $800g$ for 10 min. Preparation of nuclei was carried out at 4°C by the procedure of Krause *et al.* (1975). The cellular pellet was lysed with $80\text{mM-NaCl}/20\text{mM-EDTA}/1\%$ Triton X-100, pH 7.2, and purified nuclei were used for histone preparation by the method of Stein & Burtner (1974). The combined acid extracts were precipitated by addition of 8 vol. of acetone. The precipitates were pelleted by centrifugation at $15000g$ for 15 min, washed with acetone and then dried in a vacuum desiccator. Histones were fractionated electrophoretically on polyacrylamide gels containing 2.5M-urea by the method of Panyim & Chalkley (1969). Electrophoresis was carried out for 4 h at 2mA/gel with 0.9M-acetic acid as tray buffer. Histone gels were stained overnight in 0.1% (w/v) Amido Black in ethanol/acetic acid (20:7, v/v) and de-stained by diffusion in 0.9M-acetic acid . The amount of protein was measured spectrophotometrically at 600nm by Johns's (1967) method. For radioactivity measurement, the coloured bands were cut out, dissolved at 40°C with Soluene (Packard) and added to 10ml of scintillation 'cocktail' (Insta Gel; Packard).

Results and Discussion

The effect of different oxygen tensions on the rate of cardiac-cell growth during a 3-day period was evaluated as [$\text{Me-}^3\text{H}$]thymidine incorporation into DNA (Fig. 1a) and [$5\text{-}^3\text{H}$]uridine into RNA (Fig. 1b). At low oxygen tensions (5% , 10% $p\text{O}_2$, i.e. $\text{N}_2 + \text{O}_2 = 95:5$, $90:10$) the rate of incorporation of the two labelled precursors into nucleic acids was markedly and progressively enhanced, compared with the control oxygen tension (20% $p\text{O}_2$). In particular, both increases reached their highest measured value with 5% $p\text{O}_2$ on the third day. On the other hand, high oxygen concentrations (40% , 80% $p\text{O}_2$) caused a considerable decrease in labelled DNA and RNA, particularly evident for 80% $p\text{O}_2$ on the third day of growth (more than sevenfold). These series of experiments indicate that the adaptive capacity of cardiac cells in culture under high or low oxygen tensions is chiefly manifest in a modulation of the rate of cell proliferation. Therefore one can speculate

that oxygen has a primary effect on some mechanisms closely related to nucleic acid metabolism. The dependence of polyamine behaviour on oxygen tensions is shown in Fig. 2. The effect of low oxygen tensions results in a remarkable induction of intracellular polyamine accumulation, particularly evident for spermidine, which increases about twofold (with 5% $p\text{O}_2$) on the third day. On the other hand the high oxygen tensions cause a marked and progressive inhibition that is more evident for spermine (-95% from the control with 80% $p\text{O}_2$) rather than for spermidine (-65% at the same tension). The increase in spermidine accumulation is noteworthy because it

Fig. 1. Effect of different oxygen tensions on DNA (a) and RNA (b) synthesis in chick-embryo heart cells grown in culture for 72h

Monolayers were pulsed with [$\text{Me-}^3\text{H}$]thymidine and [$5\text{-}^3\text{H}$]uridine respectively for 2h and 1h before harvest and the rates of incorporation into DNA and RNA were measured. Each point represents the mean of four separate experiments; the s.e.m. was less than 10%. ▲, 5% $p\text{O}_2$; ○, 10%; ●, 20%; □, 40%; ■, 80%.

Fig. 2. Effect of different oxygen tensions on polyamine content in chick heart cultures during a 3-day growth period

Dansylated polyamines from the cells were separated by t.l.c. and the fluorescence intensities measured with a Turner model 111 fluorimeter equipped with a t.l.c. scanner and recorder. The data represent the contents of the polyamines spermine (Sp) and spermidine (Spd) and are expressed as percentages of the control (20% pO₂). Each point represents the mean of four separate experiments. The S.E.M. was less than 10%. A, 5% pO₂; B, 10%; C, 4%; D, 80%. ■, 1 day; □, 2 days; ▨, 3 days.

Fig. 3. Effect of different oxygen tensions on acetylation of histone fractions in cardiac-cell cultures during a 3-day growth period

Cells were exposed to 1 μ Ci of sodium [1-¹⁴C]acetate/ml for 1 h before harvest and the rate of incorporation into histone fractions was measured. Data represent the specific radioactivity of each labelled histone fraction, expressed as a percentage of the control (20% pO₂). Each point represents the mean of four separate experiments. The S.E.M. was less than 10%. ■—■, F1; □---□, F2b; ▲---▲, F2a1; ●---●, F2a2; ○---○, F3.

represents an early event associated with the process of cell replication, as observed also for neoplastic rapid-growth-stimulated systems (Bachrach *et al.*, 1967; Heby *et al.*, 1975). No experimental evidence is available on the molecular mechanism by which

oxygen modulates the intracellular polyamine content. However, since ornithine decarboxylase is an enzyme particularly sensitive to reducing agents, one might consider that high oxygen tensions are able to affect the shift of enzyme from a reduced, active,

form to an inert, oxidized, polymeric one (Jänne & Williams-Ashman, 1971).

Fig. 3 shows the changes in the rate of [$1\text{-}^{14}\text{C}$]acetate incorporation into histone fractions on increasing the tension of oxygen from 5 to 80% $p\text{O}_2$ during the 3 days of cellular growth. Low oxygen tensions produce an enhancement of histone acetylation in all the fractions, and this is particularly evident for arginine-rich histones F2a2, F2a1 and F3 during the whole experimental period. This may reflect either *N*-terminal acetylation coupled to histone synthesis or acetylation at lysine residues specific for the gene de-repression mechanism (Allfrey, 1971). Above the control $p\text{O}_2$ tension one can observe a slight decrease of radioactivity for all histone fractions, with a maximum for the arginine-rich F3 fraction. These experiments provide further evidence that the rate of cell division is closely linked with the concentration of oxygen in the environment. Similar effects of oxygen on growth of mouse fibroblasts (Brosemer & Rutter, 1961) and HeLa cells (Rueckert & Mueller, 1960) in tissue culture have been reported. The increase or impairment of thymidine and uridine incorporation into nucleic acids indicates that the earliest measured effect of oxygen occurs at, or before, the stage of transcription of RNA from DNA. The changes of the rate of [$1\text{-}^{14}\text{C}$]acetate incorporation into histone fractions observed at different $p\text{O}_2$ values could support this idea. Moreover, this modulation of the gene de-repression mechanism parallels the changes in intracellular polyamine content and nucleic acid metabolism. This is of particular interest, since spermine is able to enhance the acetylation of the arginine-rich histone fractions (Caldarera *et al.*, 1975) and to increase the RNA polymerase activity associated with nuclear chromatin (Moruzzi *et al.*, 1975). It is therefore tempting to speculate that the changes of nucleic acid metabolism and cell division induced by environmental oxygen is carried out through the modulation of intracellular polyamine content. This is in agreement with several lines of study suggesting, for the regulatory role of oxygen, a mechanism involving a process (Brosemer & Rutter, 1961) or factors (Hollenberg, 1971) closely related with cell proliferation.

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