Hypoxic upregulation of tyrosine hydroxylase gene expression is paralleled, but not induced, by increased generation of reactive oxygen species in PC12 cells

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Abstract Oxygen sensing was investigated in rat pheochromocytoma PC12 cells. They respond to hypoxia with an increased intracellular generation of reactive oxygen species (ROS), measured by oxidation of dihydrorhodamine 123. This increase is abolished by intracellular superoxide scavenging by Mn(III)-tetrakis(1-methyl-4-pyridyl)-porphyrin, and reduced or absent in the presence of the flavoprotein/complex I inhibitors, diphenyleneiodonium and rotenone. The same inhibitors, but neither intra- nor extracellular (superoxide dismutase) superoxide scavenging, abolish the hypoxia-induced increase in tyrosine hydroxylase (TH) gene expression. Thus, ROS production increases in PC12 cells during hypoxia, but this is not the cause of hypoxic TH mRNA upregulation that involves a flavoprotein.

Key words: Oxygen sensing; Tyrosine hydroxylase; Reactive oxygen species; Superoxide; Flavoprotein; PC12 cell

1. Introduction

Paraganglia, e.g. the carotid body and retroperitoneal paraganglia, serve as a defense line against hypoxia by measuring arterial oxygen supply and initiating appropriate reflex responses such as stimulation of breathing or secretion of catecholamines [1]. A tumor cell line derived from a paraganglion, the rat pheochromocytoma cell line PC12, serves as a model to investigate oxygen sensor mechanisms in paraganglionic cells. These cells respond to hypoxia by membrane depolarization [2,3], catecholamine secretion [4,5], and increased transcription and stability of mRNA for the rate-limiting enzyme of catecholamine biosynthesis, tyrosine hydroxylase (TH) [6,7]. One current theory of the molecular mechanism of paraganglionic oxygen sensing postulates a continuous production of reactive oxygen species (ROS) by a flavohemoprotein-containing NAD(P)H oxidase under normoxia, and reduced levels of ROS in hypoxia that shall trigger cellular responses [8,7], but alternative or opposing views are also held [9,10]. A critical information is whether ROS production by paraganglionic cells is influenced by hypoxia, and how ROS levels correlate to TH gene transcription. A diminished ability of PC12 cells to form hydrogen peroxide in normoxic environment immediately after exposure to hypoxia has been reported [7] but information on ROS production during hypoxia is lacking. We addressed this question by exposing PC12 cells to dihydrorhodamine 123 during 1 h periods of hypoxia. This non-fluorescent dye is oxidized by ROS to the stable, fluorescent rhodamine 123 [11], and this fluorescence was measured in fixed individual cells by laser scanning microscopy (LSM). TH mRNA was quantified in parallel experiments by densitometry of Northern/slot blots, and the influence of flavoprotein/complex I inhibitors, superoxide scavengers and a superoxide generating system on both parameters (ROS, TH mRNA) was investigated.

2. Materials and methods

2.1. Cell culture

PC12 cells were originally obtained from American Tissue Type Cell Collection (Rockville, MD, USA) and grown in RPMI 1640 medium (Sigma, Deisenhofen, Germany) containing 10% fetal calf serum (PAA, Marburg, Germany), 1% penicillin/streptomycin (PAA), 1% L-glutamine (Biomol, Hamburg, Germany) and 5% horse serum (Pan Systems, Aidenbach, Germany). They were grown and exposed to experimental conditions in 25 cm^2 flasks for mRNA analysis, and seeded on 8-well culture slides (both from Falcon, Heidelberg, Germany) 1 day before the experiment for LSM analysis of ROS production.

2.2. Messenger RNA analysis

Cells were exposed to 20% (normoxia) and 5% O_2 (hypoxia) with 5% CO_2 and N_2 as balance for 6 h, since this time point has been reported to show maximal increase in gene induction without overlaying effects on TH mRNA stability [6]. The flavoprotein/complex I inhibitors diphenyleneiodonium (DPI) and rotenone were applied at 20 μM, superoxide dismutase (SOD) at 130 U/ml (all from ICN Biomedicals GmbH, Eschwege, Germany), and the cell membrane permeable superoxide scavenger, Mn(III)-tetrakis(1-methyl-4-pyridyl)-porphyrin (MnTMPyP; Alexis, Grünberg, Germany) [12] at 50 μM. As a superoxide generating system, potassium superoxide (KO_2) was freshly dissolved in dimethylsulfoxide and applied to the cells at a final concentration of 0.25 mM. Total RNA was isolated using the RNazol reagent technique according to the manufacturers protocol (WAK Chemie, Bad Homburg, Germany), glyoxylated, electrophoresed on 1.2% agarose gel, and blotted onto nylon membrane (Nytran N13, Schleicher-Schüll, Einbeck, Germany). Blots were hybridized (for details see [13]) with digoxygenin-labeled cRNA probes specific for TH (742 bases) and β-actin [14], respectively. Digoxygenin-labeled probes were visualized by incubation of membranes for 30 min at room temperature with a 2% blocking solution (Boehringer, Mannheim, Germany) and then for 30 min at room temperature in the same solution containing a 1:10 000 (v/v) dilution of a polyclonal anti-digoxygenin sheep antibody fab fragment conjugated to alkaline phosphatase (Boehringer, Mannheim, Germany). The membranes were washed with Boehringer buffer with 0.1% Tween and alkaline phosphatase activity was determined by chemiluminescent substrate solution (CSPD) and exposure of the membranes to an X-ray film. All Northern blots revealed a single band, so that in further experiments RNA was directly applied to membranes as slots (BioRad, München, Germany), that were hybridized as before. The intensity of specific mRNA bands was quantified by densitometry (Sharp JX-

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330 color scanner, Sharp, Japan; One-Dscan, Scanalytics, Billerica, MA, USA).

2.3. ROS formation

Cells were exposed to 20% (normoxia) and 1% or 5% O_2 (hypoxia) with 5% CO_2 and N_2 as balance for 1 h in Locke solution in the presence of 25 μM dihydrorhodamine 123 (Sigma, Deisenhofen, Germany). DPI, rotenone, and MnTMPyP were applied in concentrations as described above. In five experiments (20% versus 1% O_2) the fluorescence indicator 2′,7′-dichlorofluorescein diacetate (DCFDA, 1 μM in culture medium; Sigma) was used instead of dihydrorhodamine. This dye is also oxidized by ROS yielding fluorescent 2′,7′-dichlorofluorescein (DCF) (cf. [7,15]). The culture slides were placed in an open trough with 100 ml in volume. At the end of the experiment, cells were fixed, still under specific gas exposure, by flooding the trough through an injection system with 4% paraformaldehyde in 0.1 M phosphate buffer, thereby washing-out the dye, fixing the cells, and preventing re-exposure to normoxia at the same time. Culture slides were removed, cooled to 4°C to prevent further oxidation of dihydrorhodamine due to thermically induced radical formation, and analyzed with a Zeiss confocal LSM (LSM 10, Zeiss, Jena, Germany) in non-confocal mode (×40 objective lens, zoom 2) to record the whole cells. Ten cells per well were individually traced and their fluorescence intensity recorded. One experiment included two slides exposed to normoxia and hypoxia each, and comparison of one inhibitor to control, resulting in evaluation of 40 cells (four wells) for each condition per experiment. Slides were coded so that the operator was unaware of oxygen status and experimental condition.

2.4. Data presentation

The mean optical density of slots from normoxic (20% O_2) PC12 cells hybridized with a TH-specific probe was arbitrarily set as 1, and all other values related to that. Originally, for all conditions the ratio between TH mRNA and actin mRNA was calculated, but rotenone was found to significantly increase actin mRNA which made use of the ratio meaningless. Since data showed no Gaussian distribution, they are presented as median and percentiles 25 and 75, and statistical testing was done first by Kruskal–Wallis test using SPSS software, followed by calculation of critical differences [16] or by Mann–Whitney test. Fluorescence intensities recorded by LSM could reach grey values only in the limited range between 0 and 255. Consequently, due to lack of linearity in the region of high values, a presentation of data in ratio to control values would not reflect the true differences. Thus, data are presented as pairs of control and experimental condition for each set of experiments, and were statistically evaluated by Wilcoxon test (SPSS software).

3. Results

Exposure of PC12 cells to either 1% or 5% O_2 led to a pronounced increase in intracellular rhodamine fluorescence compared to normoxia (20% O_2), that was sensitive to the intracellular superoxide scavenger, MnTMPyP (Fig. 1). Increased ROS production under 1% O_2 was also observed when DCFDA was used as fluorescent indicator instead of dihydrorhodamine (data pairs of mean fluorescence intensities in five independent experiments, normoxic/hypoxic: 104/151; 98/191; 44/196, 72/193; 19/95; $P = 0.031$). The flavoprotein inhibitor, DPI, diminished the hypoxia-induced ROS production, but the rise was still significant. On the other hand, rotenone increased ROS generation under normoxia but a further rise could not be evoked by hypoxia (Fig. 1).

We could confirm the 2-fold increase in TH mRNA after exposure of PC12 cells (at about 50% confluency) to 5% O_2 for 6 h reported previously by Kroll and Czyzyk-Krzeska [7] (Fig. 2). This hypoxia-induced increase was absent in the presence of DPI, applied either alone or in addition to MnTMPyP. Parallel to what has been observed in ROS measurements, rotenone increased TH mRNA during normoxia, but prevented a further, hypoxia-induced rise. On the other hand, neither extracellular (by SOD) nor intracellular superoxide scavenging (by MnTMPyP) suppressed increase in TH mRNA induced by 5% O_2 (Fig. 2). Application of the superoxide generator, KO_2, resulted in a trend towards decrease in TH mRNA under normoxic conditions (mean optical density normalized to normoxic control: 0.77, median = 0.78, S.E.M. = 0.09; $N = 8$) but this trend was not statistically significant ($P = 0.065$; Mann–Whitney test). The hypoxia-induced increase in TH mRNA was unaltered by KO_2 ($P < 0.01$; $N = 10$).

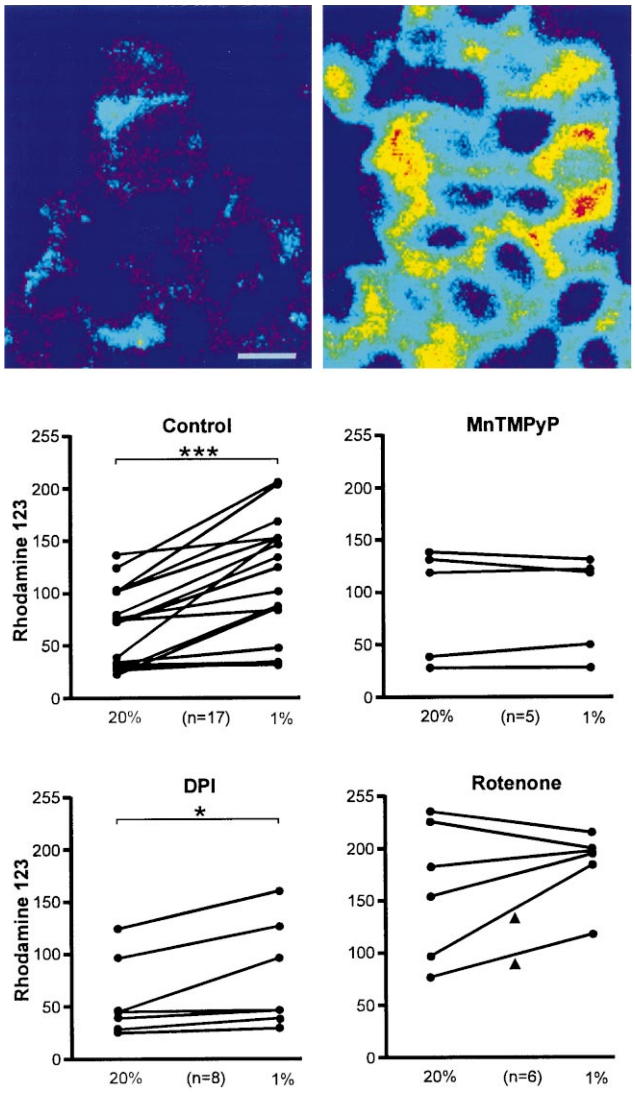

Fig. 1. Top panels: ROS production measured by oxidation of dihydrorhodamine to rhodamine, and fluorescence recording with LSM. Highest fluorescence intensity is shown in red, lowest in blue. PC12 cells exposed to 1% O_2 exhibit significantly more oxidized rhodamine than those exposed to 20% O_2. Bar = 10 μm. Lower panels: Quantification of ROS production (= fluorescence intensities). Each point represents the mean of 40 individual cells in one experiment, and is connected with its corresponding value of the same experiment, these values were statistically treated as paired data. The hypoxia-induced rise in signal is prevented by the scavenger MnTMPyP, and attenuated by DPI. Rotenone leads to a rise ($P < 0.05$) of ROS under normoxia, that made it necessary to reevaluate fluorescence in two experiments with lower gain settings at the CLSM, these data are marked with small triangles and, actually, represent the most intense fluorescence intensities recorded in these experiments. *$P < 0.05$; ***$P < 0.001$

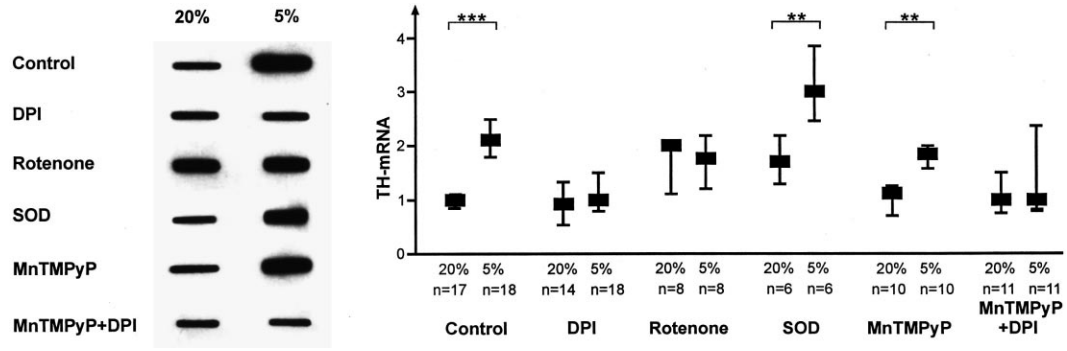

Fig. 2. Slot blot hybridized with TH mRNA-specific probe, and quantitative evaluation of optical densities of bands. The hypoxia-induced rise in TH mRNA is prevented by DPI and rotenone, albeit in the latter case at higher initial level, but unaffected by the superoxide scavengers, MnTMPyP and SOD. Data are presented as median and percentiles 25 and 75. $^{**}P < 0.01$, $^{***}P < 0.001$ (critical differences for unpaired data after global Kruskal–Wallis testing [16]).

4. Discussion

This study confirms the previously reported increase in TH mRNA in PC12 cells exposed to hypoxia, and the inhibitory effect of the flavoprotein inhibitor, DPI, on this hypoxia-induced gene transcription as reported previously from other cell lines [17], but it provides evidence against the concept of lowered generation of ROS in hypoxic PC12 cells serving as an intracellular signal regulating gene transcription. First, we observed an increase instead of the anticipated decrease in ROS production under hypoxia. At first sight this appears contradictory to what has been reported by Kroll and Czyzyk-Krzeska [7] who reported a decrease in ROS measured by DCF fluorescence. In that approach, however, cells were exposed to the fluorescent indicator after the hypoxic period while being returned to normoxic environment, whereas rhodamine oxidized during the hypoxic period was measured in the present study. An alternative explanation for the contrasting results may be the use of different indicators (DCFDA versus dihydrorhodamine) but this appears unlikely since DCF fluorescence also increased in the present experimental setup. The source of ROS generated during hypoxia in PC12 cells cannot be deduced from the present data. In Hep3B cells – a hepatoma cell line secreting erythropoietin in an oxygen-dependent manner – mitochondria have been suggested to be the source of increased ROS production under hypoxia [15]. In these cells, however, rotenone, an inhibitor of complex I of the respiratory chain, acted synergistically to DPI [15] while we observed a rise in ROS under normoxia in PC12 cells in response to rotenone. Thus, the underlying mechanisms may be different in these cells.

In principal, an increase in ROS production during hypoxia is in line with the observed inhibitory effect of DPI on hypoxic stimulation of gene transcription. DPI inhibits ROS production by the flavohemoprotein-containing NAPDH oxidase [18], and this protein complex has been detected in PC12 cells [19]. Thus, the hypothesis may be formulated that hypoxia stimulates NADPH oxidase-driven ROS production, and these ROS serve as a signal to enhance TH gene transcription. This model would explain the DPI effect, but if it were correct, ROS scavenging shall abolish the hypoxic TH mRNA induction. However, neither of the scavengers even attenuated this effect. Since both scavengers act upon superoxide [12] it might be argued that subsequent ROS such as H_2O_2 and $OH^{accent:dot}$ still have increased. However, their increase would have been detected by the dihydrorhodamine technique [20,21], which was not the case. So, effective scavenging of ROS was accompanied by unaffected increase of TH mRNA in hypoxic PC12 cells. This does not exclude the possibility that experimental induction of large amounts of ROS or their external application may well have profound effects on gene transcription. Indeed, addition of 20 μM H_2O_2 for 3–9 h to normoxic PC12 cells resulted in a specific decrease of constitutive TH mRNA expression [7]. It shall be taken into consideration, however, that decomposition of H_2O_2 generates water and oxygen, so that application of H_2O_2 can cause enormous tissue pO_2, for example 700 torr in carotid bodies bathed in 100 μM H_2O_2 solution [9]. In view of this fact, we preferred to apply a superoxide generator, KO_2, instead of hydrogen peroxide. This external application did not rise TH mRNA but rather showed a trend towards reduction, again demonstrating that increased ROS are not responsible for induction of TH gene transcription in PC12 cells.

In conclusion, PC12 cells respond to hypoxia with an increased ROS production that parallels hypoxia-induced TH gene transcription but does not cause it.

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