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Abstract

Reduced oxygenation of a variety of cells results in transcriptional upregulation of several genes, including the hematopoietic hormone erythropoietin, the angiogenic vascular endothelial growth factor (VEGF), and glycolytic enzymes such as aldolase. Recently, the heme protein cytochrome b558 of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex has been proposed as a key component of the oxygen-sensing mechanism. Cytochrome b558 consists of the p22phox and gp91phox subunits and is essential for superoxide generation in phagocytes and B lymphocytes. Mutations in these subunits result in cytochrome b558-negative chronic granulomatous disease (cytb- CGD), an inherited disorder in humans characterized by reduced microbicidal activity due to deficient superoxide generation. To test whether NADPH oxidase is involved in oxygen sensing, we exposed wild-type B-cell lines as well as cytb- CGD-derived B cell lines, deficient in either p22phox or gp91phox, to hypoxia (1% oxygen) or CoCl2 (100 mumol/L) and compared the mRNA levels of VEGF and aldolase with the untreated controls. Northern blot analysis revealed unimpaired basal and inducible expression of VEGF and aldolase mRNA in all four cytb- CGD-derived B-cell lines compared with wild-type cells. Furthermore, reconstitution of cytochrome b558 expression in cytb- CGD-derived B cells by transfection with p22phox or gp91phox expression vectors did not modify VEGF and aldolase mRNA expression. Thus, cytochrome b558 of the NADPH oxidase complex appears not to be essential for hypoxia-activated gene expression and can be excluded as a candidate for the putative universal oxygen sensor.
Hypoxic Induction of Gene Expression in Chronic Granulomatous Disease-Derived B-Cell Lines: Oxygen Sensing Is Independent of the Cytochrome b558-Containing Nicotinamide Adenine Dinucleotide Phosphate Oxidase

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Reduced oxygenation of a variety of cells results in transcriptional upregulation of several genes, including the hematopoietic hormone erythropoietin, the angiogenic vascular endothelial growth factor (VEGF), and glycolytic enzymes such as aldolase. Recently, the heme protein cytochrome b558 of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex has been proposed as a key component of the oxygen-sensing mechanism. Cytochrome b558 consists of the p22^phox and gp91^phox subunits and is essential for superoxide generation in phagocytes and B lymphocytes. Mutations in these subunits result in cytochrome b558-negative chronic granulomatous disease (cytb^- CGD), an inherited disorder in humans characterized by reduced microbicidal activity due to deficient superoxide generation. To test whether NADPH oxidase is involved in oxygen sensing, we exposed wild-type B-cell lines as well as cytb^- CGD-derived B cell lines, deficient in either p22^phox or gp91^phox, to hypoxia (1% oxygen) or CoCl2 (100 µmol/L) and compared the mRNA levels of VEGF and aldolase with the untreated controls. Northern blot analysis revealed unimpaired basal and inducible expression of VEGF and aldolase mRNA in all four cytb^- CGD-derived B-cell lines compared with wild-type cells. Furthermore, reconstitution of cytochrome b558 expression in cytb^- CGD-derived B cells by transfection with p22^phox or gp91^phox expression vectors did not modify VEGF and aldolase mRNA expression. Thus, cytochrome b558 of the NADPH oxidase complex appears not to be essential for hypoxia-activated gene expression and can be excluded as a candidate for the putative universal oxygen sensor.

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vation in a naturally occurring model of cytochrome b558 deficiency, using B-cell lines derived from patients with cytochrome b558-negative chronic granulomatous disease (cyt b CGD). In this inherited disorder, NADPH oxidase activity is absent or drastically reduced due to defects in the p22phox (autosomal cyt b [A22phox]) and the gp91phox (X-linked cyt b [X91phox]) subunit of cytochrome b558. Assuming that cytochrome b558-derived H2O2 was regulating hypoxia-inducible gene expression, B-cell lines derived from cyt b CGD patients would exhibit increased constitutive expression of hypoxia-inducible genes compared with B-cell lines derived from healthy donors. Moreover, if cytochrome b558 were a central component of the oxygen sensor, cyt b CGD-derived B-cell lines would exhibit defective sensing in hypoxia. Consequently, hypoxia-inducible genes would no longer be activated by lowering the oxygen concentration. Reconstitution of the cytochrome b558-dependent oxidase activity in cyt b CGD B-cell lines by transfection with expression constructs containing the corresponding oxidase components should restore a defect in the putative oxygen sensor. To investigate the oxygen-sensing capabilities of these cell lines, we chose to determine the basal and inducible steady-state mRNA levels of the oxygen-regulated genes VEGF, a potent inducer of angiogenesis leading to vascularization of hypoxic tissue, and the glycolytic enzyme aldolase, needed for aerobic as well as anaerobic glycolysis, which represents the main energy source under hypoxic conditions.

**MATERIALS AND METHODS**

**Epstein-Barr virus (EBV)-transformed B-lymphocyte cell lines (B-cell lines).** B-cell lines were obtained by transformation of peripheral blood lymphocytes with EBV as described and were maintained in a humidified incubator containing ambient air and 5% CO2 at 37°C in RPMI 1640 medium (GIBCO-BRL, Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), penicillin, streptomycin, and ciprofloxacin, buffered to pH 7.2 with 20 mmol/L HEPES. Viability of EBV-transformed cell lines was routinely over 90% as tested by trypan blue exclusion. The two wild-type B-cell lines WT:1 and WT:2 were derived from normal blood donors and express cytochrome b558. The two unrelated autosomal cyt b CGD-derived B-cell lines A22phox;1 and A22phox;2 with the primary defect in p22phox, and reconstitution of their NADPH oxidase activity by transfection with expression plasmids encoding p22phox (pEBOp22), have been described earlier. The two X91phox;1 and X91phox;2 B-cell lines were derived from unrelated patients with the most common cyt b X-linked form of CGD, namely, a defect in the gp91phox subunit. The X91phox;1 line was transfected either with the expression vector pEBO alone or with the vector pEBOgp91, directing expression of gp91phox as described. Chemiluminescence monitoring of H2O2 production of cell lines was performed with 100 ng/mL phorbol myristate acetate (PMA) as described.

**Hypoxic induction of B-cell lines.** Routinely, exposure to low oxygen was performed with 1.5 × 107 cells. After replacing the medium with 15 mL high glucose Dulbecco's modified Eagles medium (DMEM; GIBCO-BRL) containing 10% FCS (Boehringer Mannheim, Roche, Switzerland), penicillin, streptomycin, 1x minimal essential medium (MEM) nonessential amino acids, 2 mmol/L L-glutamine, and 1 mmol/L Na-pyruvate (all GIBCO-BRL), the cells were incubated for 16 hours or 65 hours at either 20% O2 (140 mm Hg, normoxia) or 1% O2 (7 mm Hg, hypoxia), or with 100 μmol/L CoCl2 in a Forma Scientific incubator (Model 3319 Forma Scientific Incubator; Brouwer, Luzern, Switzerland).

Quantitative Northern blot analysis. Immediately after stimulation, RNA was isolated from the B-cell lines as described by Chomczynski and Sacchi. Total RNA (20 μg) was denatured in formaldehyde and electrophoresed through a 1% agarose gel containing 6% formaldehyde as described. After pressure blotting (Stratagene, Zürich, Switzerland) to nylon membranes (Biodyne A; Pall, Winiger, Wohlen, Switzerland) and ultraviolet (UV) crosslinking (Stratalinker; Stratagene), the filters were hybridized to cDNA probes labeled with α32P-deoxyctydine triphosphate (dCTP) to a specific activity of 1 × 109 dpm/μg using the random primed DNA labeling method. Hybridization was performed in 50% formamide, 10% dextran sulfate, 5x Denhardt's solution, 200 μg/mL sonicated salmon sperm DNA, 1% sodium dodecyl sulfate (SDS), 0.9 mol/L NaCl, 60 mmol/L NaH2PO4, 6 mmol/L EDTA (pH 7.0) for 14 hours at 42°C. The filters were washed to a final stringency of 55°C in 0.1x saline sodium citrate (SSC), 0.2% SDS, and the signals were recorded and quantified using a PhosphorImager (Molecular Dynamics, Paul Bucher, Basel, Switzerland). The VEGF cDNA probe pmVh was obtained by subcloning a reverse transcription-polymerase chain reaction (RT-PCR) amplification product derived from mouse brain RNA using the primers 5'-gggacctGCGGGTCGCTCCAGTGC-3' (sense) and 5'-gggacctGCACGGCGCTTGGCTTGTCAC-3' (antisense), which span the entire coding region. The fructose-1,6-bisphosphate aldolase A (aldolase) cDNA probe p30 and the ribosomal protein L28 cDNA probe p14.1 were cloned from an HepG2 cDNA library (C.C.S.-M. and R.H.W., unpublished work, July 1994). All probes were purified free of vector sequences by restriction digestion and agarose gel purification. VEGF and aldolase mRNA signals were normalized to the signal obtained with the L28 cDNA probe to correct for loading and blotting differences. In contrast with the generally used normalization probes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were found to be affected by hypoxia in most cell lines studied, L28 mRNA levels were not or were only marginally influenced by hypoxia (R.H.W. and C.C.S.-M., unpublished work, February 1995).

**Statistical analysis.** (1) For hypoxia-inducible expression of VEGF and aldolase in cyt b B-cell lines (ie, WT:1, WT:2, A22phox;1/p22, A22phox;2/p22, and X91phox;1/gp91), steady-state mRNA levels (estimated as described above) were expressed as percent increase over the corresponding untreated control. Values are given as means ± SEM of n estimations. To test the significance of changes, the one-sample Wilcoxon test was applied, and P < .05 was considered as statistically significant. (2) For constitutive expression of VEGF and aldolase mRNAs, steady-state mRNA levels within each experiment were expressed as a percentage of the result obtained with the wild-type cell line WT:1, which was present on each Northern blot. This internal normalization control was necessary, because data from different Northern blots were pooled. Descriptive statistics (mean ± SEM) were calculated for a group of cyt b cell lines (ie, WT:2, A22phox;1/p22, A22phox;2/p22, and X91phox;1/gp91) and the group of cyt b cell lines (ie, A22phox;1/A22phox;2/vector, A22phox;2/vector, X91phox;1, X91phox;2/vector, and X91phox;2). To analyze statistically significant differences between the two groups, the nonparametric U test according to Mann-Whitney-Wilcoxon was performed. (3) For hypoxia and CoCl2-stimulated expression of VEGF and aldolase mRNA, steady-state mRNA levels were expressed as percent increase over the corresponding untreated control present on the same Northern blot. Descriptive statistics (mean ± SEM) were calculated for the group of cyt b cells defined in (1) and cyt b cells defined in (2). As above, statistically significant differences between two groups were assessed by the nonparametric U test.

**RESULTS**

Expression of VEGF and aldolase in human B-cell lines is inducible by hypoxia and CoCl2. Provided that hypoxia-
inducible gene activation can be demonstrated in B cells, genetically cytochrome b558-deficient CGD B cell lines offer the possibility to examine directly the hypothesis that cytochrome b558 of NADPH oxidase is involved in oxygen sensing. Most data leading to this hypothesis have been generated by determining EPO production in human hepatoma cell lines and by electrophysiologic measurements in oxygen-sensing neuronal cells, both of which are not feasible in B-cell lines. However, the genes encoding VEGF and the glycolytic enzymes have been demonstrated to be hypoxia- and CoCl₂-inducible in a variety of cell lines with a high degree of similarity to EPO induction. We, therefore, investigated hypoxia- and CoCl₂-inducible regulation of VEGF and aldolase mRNA in B-cell lines with the aim of establishing an alternative cellular model to study regulation of gene expression by these stimuli. Several wild-type, CGD-derived, and oxidase-reconstituted transfected B-cell lines were used, as described in Materials and Methods. Northern blots were performed to analyze VEGF and aldolase steady-state mRNA levels in B-cell lines that had been exposed to either 20% oxygen (normoxia), 1% oxygen (hypoxia), or 100 μmol/L CoCl₂. Cobalt was included because it mimics the effects of hypoxia in most systems so far investigated. VEGF and aldolase mRNA were constitutively expressed in all B cell lines tested (Fig 1A through C). Hypoxic increase of VEGF and aldolase mRNA in cytb⁺ cells over normoxic control cells was 140% ± 21% and 73% ± 13%, respectively (n = 7, P < .05, Wilcoxon test). CoCl₂-induced increase of VEGF and aldolase mRNA in these cells was 77% ± 19% and 49% ± 13%, respectively (n = 7, P < .05, Wilcoxon test). At present, the role of VEGF expression in lymphocytes is an open question and may have relevance for angiogenesis associated with immune reactions and with lymphocyte-derived tumors. Nevertheless, this novel finding provided the tool to investigate the role of NADPH oxidase in oxygen sensing.

Lack of H₂O₂ production in CGD-derived B-cell lines. Before commencing further studies, we verified NADPH oxidase activity of the wild-type, CGD-derived, and oxidase-reconstituted transfected B-cell lines used. Chemiluminescence monitoring of H₂O₂ production by these cell lines after PMA stimulation yielded the following values (integrated counts × 10⁻⁵ over 4 hours, means of triplicates with SE less than 15%): WT:1, 9.335; WT:2, 6.126; A22²⁺:1, 142; A22²⁺/p22, 895; A22²⁺/vector, 148; A22²⁺/2, 135; A22²⁺/2/p22, 1,488; A22²⁺/vector, 152; X91¹⁻/1, 128; X91¹⁻/1/gp91, 1,214; X91¹⁻/vector, 146; X91¹⁻/2, 138; background, 144. Unless stimulated, no H₂O₂ production over the background level of the chemiluminescence assay could be demonstrated in all cell lines. Thus, parental and vector-only transfected cytb⁺ CGD-derived B-cell lines exhibited no detectable H₂O₂ generation, while wild-type and cytochrome b558-reconstituted cytb⁺ B-cell lines were actively producing H₂O₂ if stimulated with PMA.

Basal VEGF and aldolase mRNA levels are independent of a functional cytochrome b558 NADPH oxidase. It has been postulated that H₂O₂ suppresses mRNA levels of hypoxia-inducible genes, suggesting that enhancement of mRNA levels of such genes observed under hypoxia is mediated by diminished generation of H₂O₂ via NADPH oxidase. If this relief-from-suppression model were correct, one might expect that cytb⁻ CGD-derived B-cell lines would exhibit increased basal VEGF and aldolase expression already under normoxic conditions. As shown in Fig 1, cytb⁻ CGD-derived B-cell lines displayed similar basal VEGF and aldolase mRNA levels as cytb⁺ B cells (ie, wild-type and reconstituted cell lines), implying that expression of both genes was not altered in cytb⁻ cells. This observation was verified by collectively comparing the VEGF and aldolase mRNA levels present in cytb⁻ cells to the values obtained from cytb⁺ cell lines. VEGF mRNA levels, expressed as a percentage of the level found in the wild-type line WT:1 (mean ± SEM of n determinations), were 55% ± 38% (n = 4) for cytb⁻ lines and 72% ± 24% (n = 10) for cytb⁺ lines, respectively, with no statistically significant difference detectable (U test, P > .05). Likewise, no significant difference between levels of aldolase mRNA in the group of cytb⁻ cell lines (100% ± 13%) and the group of cytb⁺ lines (80% ± 4%) was apparent (U test, P > .05). These results strongly support the notion that cytochrome b558 NADPH oxidase-derived H₂O₂ is unlikely to be involved in the regulation of oxygen-sensitive genes. However, because the H₂O₂ concentration of unstimulated wild-type B cells, as well as PMA-stimulated cytb⁻ CGD B cells, was below the detection limit of the chemiluminescence assay (see above), we were unable to determine whether there was any difference in basal H₂O₂ production between these two cell types. Thus, hypoxic stimulation experiments were necessary to clearly establish whether the cytochrome b558-dependent NADPH oxidase is involved in oxygen-sensing.

Cytochrome b558 NADPH oxidase is not involved in sensing hypoxia. Three sets of induction experiments were performed, as shown in Fig 1. First, the cytb⁺ wild-type cell line WT:1 and the cytb⁻ CGD-derived cell lines A22²⁺/1, A22²⁺/2, and X91¹⁻/1 were exposed for 16 hours to either 20% O₂, 1% O₂, or 100 μmol/L CoCl₂ (Fig 1A). Second, to exclude cell line-specific variations that might confound the results, we examined cytb⁻ CGD lines that were transfected with expression vectors directing synthesis of the corresponding intact cytochrome b558 subunits that reconstituted NADPH oxidase activity. Reconstituted cell lines (A22²⁺/1/p22, A22²⁺/2/p22 and X91¹⁻/1/gp91), vector-only transfected cell lines (A22²⁺/1/vector, A22²⁺/2/vector and X91¹⁻/1/vector), and the wild type line WT:1 were induced for 16 hours (Fig 1B). Third, a prolonged induction experiment (65 hours) was performed using two additional B-cell lines: the wild-type line WT:2 and the gp91²⁻/²⁻/-deficient line X91¹⁻/2 (Fig 1C).

Steady-state mRNA levels were determined by quantitative Northern blot analysis. As shown in Fig 2, hypoxia and CoCl₂ not only substantially increased VEGF and aldolase mRNA levels in the wild-type and reconstituted cytb⁺ B-cell lines, but also in cytb⁻ CGD-derived lines. The hypoxia-induced elevation over normoxic controls in cytb⁻ lines ranged from 74% to 246% for VEGF and 29% to 122% for aldolase (Fig 2) and was not quantitatively impaired in the cytb⁻ CGD lines. Overall, hypoxic VEGF mRNA induction was somewhat lower than previously published for other cell lines. However, as the degree of hypoxic induction of
VEGF varies from cell line to cell line, different maximal VEGF induction in B cells compared with other cell lines (and even among different B-cell lines) is not surprising. In contrast, hypoxic aldolase induction was within the published increase in transcription rates after 24 hours of hypoxia in skeletal muscle cells. When comparing collectively all data from cyt b<sup>−</sup> lines with those from cyt b<sup>+</sup> lines, steady-state VEGF mRNA levels, expressed as percent increase over the normoxic control (mean ± SEM of n determinations), were 140% ± 21% (n = 7) and 129% ± 15% (n = 10), respectively, with no statistically significant difference detectable (U test, P > .05). The same was true for aldolase mRNA, where the values were 73% ± 13% for cyt b<sup>−</sup> lines and 91% ± 13% for cyt b<sup>+</sup> lines, respectively. Responses to CoCl<sub>2</sub> stimulation were lower than for hypoxic induction. However, comparison of all data from cyt b<sup>−</sup> lines with those from cyt b<sup>+</sup> lines (Fig 2), expressed as percent increase over the CoCl<sub>2</sub>-free normoxic controls (mean ± SEM), revealed no statistically significant difference (U test, P > .05). The values for VEGF were 77% ± 19% (n = 7) for cyt b<sup>−</sup> lines and 79% ± 12% (n = 10) for cyt b<sup>+</sup> lines, respectively. The same was true for aldolase, where the values were 49% ± 13% and 71% ± 9%, respectively. Collectively, these data indicate that absence of cytochrome b558 had no significant effect on and, in particular, was not abrogating mRNA induction of VEGF and aldolase by either hypoxia or CoCl<sub>2</sub>.

**DISCUSSION**

The results presented in this report demonstrate that, notwithstanding some cell line-dependent variation, each of the four cyt b<sup>−</sup> CGD lines examined is capable of hypoxic and CoCl<sub>2</sub>-driven induction of two oxygen-regulated genes. This indicates that cytochrome b558 has no effect on, and especially is not necessary for, hypoxia- or CoCl<sub>2</sub>-stimulated induction of gene expression. However, we cannot formally exclude the possibility that a reduction in cytochrome b558-independent H<sub>2</sub>O<sub>2</sub> production, at concentrations not detectable with the chemiluminescence assay, is involved in sensing low oxygen concentrations.

At least with regard to hypoxic induction, our conclusion
is in line with a recent publication demonstrating that the flavoprotein inhibitor diphenylene iodonium chloride (DPI), a known inhibitor of NADPH oxidase activity, had no effect on hypoxic EPO induction in Hep3B cells at 30 nmol/L. Interestingly, this concentration reduced the CoCl₂ induction of EPO to about 50%. Our results suggest that this inhibitory mechanism was not due to an inhibition of NADPH oxidase, but was probably the result of interactions with other, yet-to-be-defined targets. Indeed, micromolar concentrations of DPI are necessary for NADPH oxidase inhibition in phagocytes and B cells, but DPI has been reported to affect other flavoprotein-dependent enzymes such as nitric oxide synthetase, even at nanomolar concentrations. Therefore, apparent mimicry of hypoxia by CoCl₂ might involve more components than the oxygen sensor, one of them probably being a DPI-sensitive flavoprotein but not a component of the cytochrome b558 of NADPH oxidase. Interpretation of studies using DPI to inhibit NADPH oxidase may be limited by the unspecificity of DPI. Our genetic approach, in contrast, directly and specifically rules out cytochrome b558 as a candidate for a universal oxygen sensor. Finally, in vivo observations of patients with CGD and gp91phox-deficient mice that display reduced microbicidal activity and that do not reveal obvious signs expected from defects in oxygen-sensing, like impaired erythropoiesis or angiogenesis, further support our conclusion that cytochrome b558 has no relation to the putative oxygen sensor.

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