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Hydrogen Peroxide- and Cell-Density-Regulated Expression of NADH-Cytochrome b_5 Reductase in HeLa Cells

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Environmental conditions regulate the expression of different antioxidant enzymes in cell culture. We have studied the effect of cell density and hydrogen peroxide on the expression of NADH-cytochrome b_5 reductase in HeLa cells. Polypeptide levels of the NADH-cytochrome b_5 reductase increased about three fold in confluent HeLa cells compared to sparse cells. Addition of H_2O_2 to HeLa cells altered expression levels of the NADH-cytochrome b_5 reducatase in a concentration-dependent way, being sparse cells more sensitive to H_2O_2 addition than confluent cells. The presence of pyruvate, a H_2O_2 scavenger, produced a significant increment (200%) in the levels of NADH-cytochrome b_5 reductase in sparse cells, but less increase (25%) in confluent cells, suggesting that generation of endogenous H_2O_2 could repress NADH-cytochrome b_5 reductase expression, particularly in sparse cultures. Accordingly, confluent HeLa cells showed significantly lower levels of reactive oxygen species than cells in sparse cultures. Addition of tert-butylhydroquinone, a compound which generates reactive oxygen species through redox cycling, also reduced expression of the NADH-cytochrome b_5 reductase. Increments in several antioxidant enzymes taking place during confluency could participate in the increase of NADH-cytochrome b_5 reductase expression by reducing reactive oxygen species levels in cells. Overall, our results support that acute oxidative stress caused by H_2O_2 inhibits the expression levels of NADH-cytochrome b_5 reductase, most likely due to inhibition of SP1 transcriptional activity. On the other hand, adaptation to H_2O_2 involved increased expression of the cytochrome b_5 reductase, supporting the existence of additional regulatory mechanisms.

KEY WORDS: Antioxidant enzymes; reactive oxygen species; NADH:cytochrome b_5 reductase; hydrogen peroxide; cell density; HeLa cells.

NADH-cytochrome b_5 reductase (NADH:ferricytochrome b_5 oxidoreductase, E.C. 1.6.2.2) (Cytb_5R) is an integral membrane enzyme located mainly in the endoplasmic reticulum and outer mitochondrial membranes (Borgese and Pietrini, 1986), but at minor extent also in plasma membranes (Remacle, 1980), with a large cytosolically exposed catalytic domain and a short *N*-terminal membrane tail anchor (Ozols *et al.*, 1985). Soluble forms in erythrocytes and nonerythriod cells have been described as well (Hultquist *et al.*, 1978; Leroux *et al.*, 2001). Biochemical and molecular studies have shown that Cytb_5R in its various subcellular and tissue locations derives from the translation of various types of transcripts generated by the use of alternative promoters and splicing pattern (Leroux *et al.*, 2001; Pietrini *et al.*, 1988, 1992).

Classical functions described for this enzyme are desaturation and elongation of fatty acids (Keyes and Cinti, 1980; Oshino *et al.*, 1971), cholesterol biosynthesis (Reddy *et al.*, 1977), drug metabolism (Hildebrandt and Estabrook, 1971), and methemoglobin reduction in erythrocytes (Hultquist and Passon, 1971). New and important emerging functions for Cytb_5R are the regeneration of both hydrophilic and lipophilic antioxidants. In this way, the molecular approach has demonstrated that

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this enzyme participates in the regeneration of ascorbate from ascorbate-free radical (Shirabe *et al.*, 1995), confirming previous biochemical evidence that suggested a role in reduction of the ascorbate-free radical via an outer mitochondrial-membrane-specific isoform of cytochrome b_5 (Diliberto *et al.*, 1982; Ito *et al.*, 1981). Very recent molecular studies in the yeast model *Saccharomyces cerevisiae* have demonstrated a crucial role for mitochondrial Cytb_5R in the reduction of D-erythroascorbyl free radical, that is however fully independent of cytochrome b_5 (Lee *et al.*, 2001). In addition, plasma membrane Cytb_5R can function as a quinone reductase reducing ubiquinone in situ or in reconstituted liposomes (Navarro *et al.*, 1995; Villalba *et al.*, 1997). In turn, ubiquinol can reduce external ascorbate-free radical to ascorbate (Gómez-Díaz *et al.*, 1997; Santos-Ocaña *et al.*, 1998a,b; Villalba *et al.*, 1995). The lipophilic antioxidant vitamin E can also be regenerated by the Cytb_5R system in both mitochondria and plasma membranes (Constantinescu *et al.*, 1993, 1994). Thus, together with other quinone reductases, a role for Cytb_5R may be the maintenance of appropriate levels of antioxidants, and this enzyme should be recognized as basic in protecting membrane lipids from peroxidation.

Many metabolic reactions generate free radicals, mainly reactive oxygen species (ROS), whose levels must be strictly controlled to avoid serious damage to cellular structures. Cells undergoing oxidative stress respond increasing their antioxidant defense system that, among others, includes glutathione peroxidase, catalase, CuZn superoxide dismutase, Mn-dependent superoxide dismutase, NAD(P)H:quinone oxidoreductase (NQO1), ubiquinone, Cytb_5R, and the activation of oxidative stress-responsive transcription factor NF-$\kappa\beta$ (Bello *et al.*, 2001; Doostdar *et al.*, 1990; Forthoffer *et al.*, 2002; Franco *et al.*, 1999; Kim *et al.*, 2001; Meyer *et al.*, 1993; Moon *et al.*, 2001; Navarro *et al.*, 1998; Zhou *et al.*, 2001). Cultured cells modify the expression pattern of antioxidant enzymes not only in response to exogenous oxidative stress (Duthie and Collins, 1997; Kasugai and Yamada, 1992; Pinkus *et al.*, 1996; Röhrdanz and Kahl, 1998; Tome and Briehl, 2001), but also during ageing in culture because ROS are endogenously generated by cells and released to culture medium (Carrera-Rotllan and Estrada-Garcia, 1998; Moon *et al.*, 2001; Piotrowski *et al.*, 2000). Cultured cells also modulate their antioxidant systems according to the growth phase, where an increase in superoxide dismutase activity in 3T3 fibroblast cells (Oberley *et al.*, 1995) and melanoma cell lines (Bravard *et al.*, 1999) has been described at the time when the cells stop proliferation. Also, a cell-density-related increase in NQO1 activity has been reported in normal 3T3 fibroblasts (Schlager *et al.*, 1993), human osteoblastic cells (Collin *et al.*, 2001), and HeLa cells (Bello *et al.*, 2001), and this regulation of NQO1 activity is dependent on H_2O_2 (Bello *et al.*, 2001).

We have studied in this work the expression of Cytb_5R in cell cultures at different densities and the effect of H_2O_2, because this enzyme participates in the cell antioxidant defense but it is generally considered as a constitutive housekeeping enzyme (Zenno *et al.*, 1990). Our results show a significant increase of Cytb_5R polypeptide in confluent cells compared to sparse cells. In addition, Cytb_5R is increased in cells adapted to chronic exposure to H_2O_2. However, both acute oxidative stress induced by H_2O_2, and ROS produced by redox cycling of *tert*-butylhydroquinone (TBHQ) decreased its expression. Cell-density-related expression pattern of Cytb_5R is explained on the basis of a decrease in the levels of ROS at confluency. The involvement of complex transcriptional mechanisms that could regulate expression of Cytb_5R under oxidative stress is discussed.

MATERIAL AND METHODS

Cell Cultures

Cultures of HeLa cells were maintained at the laboratory in Dubelcco's MEM (Sigma) supplemented with 10% fetal calf serum (Flow Laboratories), 100 units/mL penicillin, 100 mg/mL streptomycin and 2.5 mg/mL amphotericin B (Sigma) at 37°C in a humified atmosphere of 5% CO_2 and 95% air. Prior to experiments using H_2O_2, the cells were transferred to iron and pyruvate-free MEM (Sigma) (Meyer *et al.*, 1993). Cells were cultured on 50 cm^2 culture dishes at 1500 and 25,000 viable cells/cm^2 and then grown until a density of about 8000 (low density, sparse) or 100,000 (high density, confluence) viable cells/cm^2 respectively was reached. To study the levels of Cytb_5R at increasing densities, HeLa cells were cultured at 1500 viable cells/cm^2 and then grown for 6 days. In this case, the culture medium was changed every 2 days. In some experiments, 10 mM sodium pyruvate was added to the culture medium to study the effect of H_2O_2 scavenging (Giandomenico *et al.*, 1997). For preparing membrane fractions (see below), cells were separated from dishes using a nonenzymatic cell dissociation solution (Sigma).

Cultures of HL-60 cells were maintained in RPMI (Bio Whittaker Europe) supplemented with 10% fetal calf serum (Flow Laboratories), 100 units/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B (Sigma) at 37°C in a humified atmosphere of 5% CO_2 and 95% air. Adaptation of HL-60 cells to H_2O_2 was carried out basically according to the protocol described

by Kasugai and Yamada (1992). Briefly, the cells were initially seeded at a density of 5×10^5 viable cells/mL and exposed to 100 μM H_2O_2 for 2–7 days. After exposure, the cells were harvested and seeded in fresh medium without H_2O_2 for 3–14 days. After recovery, the cells were again exposed to 100 μM H_2O_2. The cell density for seeding was gradually decreased (5, 4, 3, 2, 1.5×10^5 viable cells/mL) as the time required for recovery of growth was shortened. After an adaptation period of 4 months, cells could grow without any delay when inoculated at 1.5×10^5 viable cells/mL in culture medium containing 100 μM H_2O_2. Once adapted, cells were maintained at the laboratory with culture changes and addition of fresh 100 μM H_2O_2 every 2 days. In all cases, the viability of cell cultures was estimated by tripan blue exclusion assay.

Preparation of Membrane Fractions

All procedures were carried out at 4°C. Cells were concentrated by centrifugation at $1000 \times g$ for 5 min, and washed with cold 130 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 0.1 mM dithiothreitol, and 1 mM PMSF. The cells were centrifuged again and resuspended in 1 mL of hypotonic lysis buffer: 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM PMSF, and 20 μg/μl each of chymostatin, leupeptin, antipain, and pepstatin A (CLAP). Homogenization of cells was carried out for 5 min with the aid of a glass–glass potter and then for 5 s with a mechanical cell homogenizer. After lysis of cells, the same volume of 250 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM PMSF and CLAP was added to restore osmolarity. Unbroken cells were separated by centrifugation at $800 \times g$ for 5 min and the supernatant was saved. Membranes were separated from cytosolic fractions by ultracentrifugation at $100{,}000 \times g$ for 30 min.

SDS-PAGE, Western Blotting, and Cyt*b*5R Quantification

Prior to electrophoresis, membrane fractions were suspended in SDS-dithiothreitol loading buffer (10% sucrose, 2 mM EDTA, 1.5% (w/v) SDS, 20 mM dithiothreitol, 0.01% (w/v) bromophenol blue, and 60 mM Tris-HCl (pH 6.8)). Samples were separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose sheets. Blots were stained with Ponceau-S for visualization of total protein and then stained with a polyclonal antibody raised against Cytb_5R purified from pig liver membranes (Navarro *et al.*, 1995) and a secondary antibody coupled to alkaline phosphatase (Sigma). Quantification (in arbitary units) was performed with Quantiscan software (Biosoft, U.S.A.) both in Ponceau-S-stained lanes and in specifically antibody-stained Cytb_5R bands. Reaction intensity of antibody-stained bands was normalized to that of the corresponding lane stained with Ponceau-S to correct for possible differences in protein loading between samples (Forthoffer *et al.*, 2002).

Measurement of Reactive Oxygen Species

Levels of peroxide and superoxide in cells were quantified using the probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (Het) respectively (Bindokas *et al.*, 1996; Oyama *et al.*, 1994). Cells were incubated in the dark with the corresponding probe (final concentrations: 10 μg/mL DCFH-DA and 4 μM Het) for 30 min at 37°C. After washing, cells were detached from cultured plates and fluorescence was measured by flow cytometry using a Coulter EPICS X L cytometer equipped with a 488-nm Argon laser. Fluorescence was determined at 525 nm (FL1) for fluorescein, and at 620 nm (FL3) for ethidium.

RESULTS

Quantification of Cytochrome b_5 Reductase Polypeptide

Levels of Cytb_5R polypeptide were quantified by immunostaining with a polyconal antiserum raised against purified pig liver protein (Navarro *et al.*, 1995), and a secondary antibody coupled to alkaline phosphatase. The specific antiserum recognized only one major band of about 36 kDa in blots of membrane proteins obtained from HeLa cells (Fig. 1(A)), which agrees with an identical staining pattern of pig liver membrane proteins (Navarro *et al.*, 1995). To estimate the linear range of detection which allowed us to carry out a reliable quantification of Cytb_5R, a calibration with increasing amounts of membrane protein was performed before this method was used to quantify levels of Cytb_5R polypeptide in the samples. As shown in Fig. 1(A)–(C), reaction density was linear between 10 and 40 μg of membrance protein, but higher protein loading in electrophoresis gels resulted in a loss of linearity of Cytb_5R band staining. Therefore, quantifications of Cytb_5R were carried on with 30 μg of membrane protein, a concentration that is in the linear range, thus allowing to detect both increases and decreases in the expression of this enzyme.

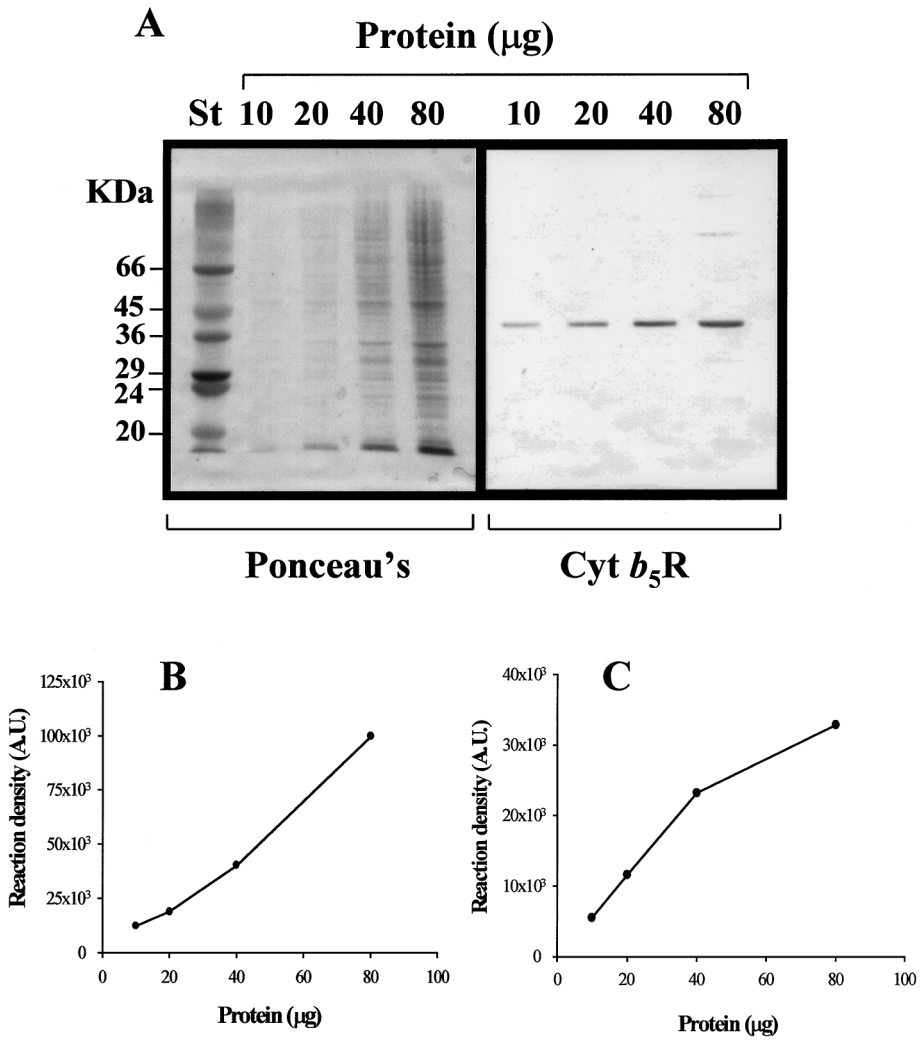

Fig. 1. Quantification of cytochrome b_5 reductase by antibody staining in western blots of HeLa cell membranes. (A) Western blot stained with Ponceau-S (left panel) and anti-cytochrome b_5 reductase antiserum (right panel). Molecular masses of standards (St) are indicated. Each lane of the gel was loaded with 10–80 μg of membrane proteins from HeLa cells as indicated. After electrophoresis and blotting, nitrocellulose sheets were stained with Ponceau-S, images were captured and blots were then destained for reaction with specific antiserum against cytochrome b_5 reductase. After developing of bands, images were captured for quantification. (B) Quantification (arbitrary units) of staining with Ponceau-S. (C) Quantification (arbitrary units) of staining with cytochrome b_5 reductase antiserum.

Role of Cell Density on Cytochrome b_5 Reductase Levels in HeLa Cells

For these experiments HeLa cells were plated at 1500 cells/cm^2 on 50 cm^2 culture dishes in MEM supplemented with 10% fetal calf serum. After 3 days growing, cells reached a density of about 8000 cells/cm^2 (sparse culture). Separate dishes were allowed to grow within the exponential phase for another three additional days until HeLa cells reached confluency (about 90,000 cells/cm^2, dense culture) (Fig. 2(A)). Membrane fractions were obtained every day and used for Cytb_5R quantifications. Levels of Cytb_5R polypeptide in cell membranes increased about three fold between days 3 and 5 of culture, when cell density reached about 40,000 cells/cm^2 (subconfluent culture). Afterwards, the amount of Cytb_5R polypeptide showed a slight decline when cell culture became confluent (Fig. 2(B)). Since most Cytb_5R increase occurred before confluency, this data suggests that some diffusible factor could be responsible of the rise of Cytb_5R levels rather than cell-to-cell contacts.

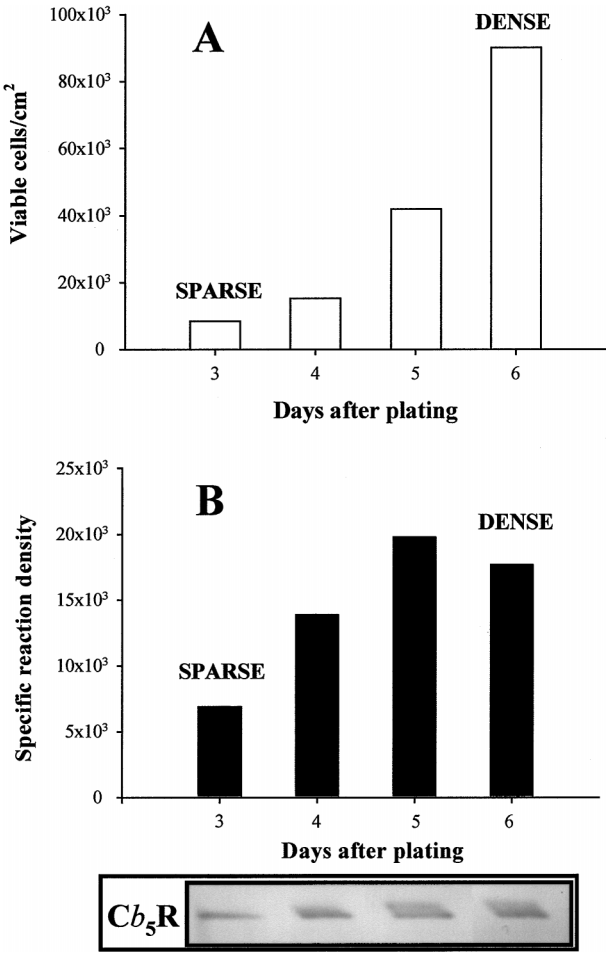

Fig. 2. Role of cell density in the levels of cytochrome b_5 reductase polypeptide in HeLa cells. (A) Time course of cell density. (B) Cytochrome b_5 reductase polypeptide increase. Cells were grown either under standard conditions in MEM containing 10% serum. At the indicated times, cells were detached from culture plates and the number of viable cells counted. Membrane fractions were obtained and proteins were electrophoresed, blotted onto nitrocellulose and immunostained. Results obtained after quantification of antibody-stained bands were normalized to density reaction of the corresponding lanes stained with Ponceau-S and expressed in arbitray units. Cell density was calculated as the number of viable cells/cm^2. A representative western blot showing inmunostained cytochrome b_5 reductase band in membranes from cells at increasing densities is also depicted in panel B. Results presented are representative of three independent experiments.

Role of ROS in Cell-Density-Regulated Expression of Cytochrome b_5 Reductase

We recently found that activity of NQO1 increased up to 30-fold with cell density, this effect being mediated partly by endogenous generation of H_2O_2 (Bello *et al.*, 2001). To study whether or not H_2O_2 was also involved in the regulation of Cytb_5R expression, we first evaluated the effect of exogenous H_2O_2 addition on Cytb_5R levels in both sparse and confluent cultures of HeLa cells. For these experiments, we used concentrations of H_2O_2 ranging between 1.5 and 24 μM that produced no significant losses of cell viability with respect to control cells at any tested cellular density (Bello *et al.*, 2001). In sparse HeLa cells, exogenous H_2O_2 decreased the basal levels of Cytb_5R polypeptide in a dose-dependent manner, reaching a plateau of 66% inhibition from a concentration of 6 μM H_2O_2 (Fig. 3(A)). In confluent cultures, addition of H_2O_2 at concentrations ranging from 1.5 to 12 μM

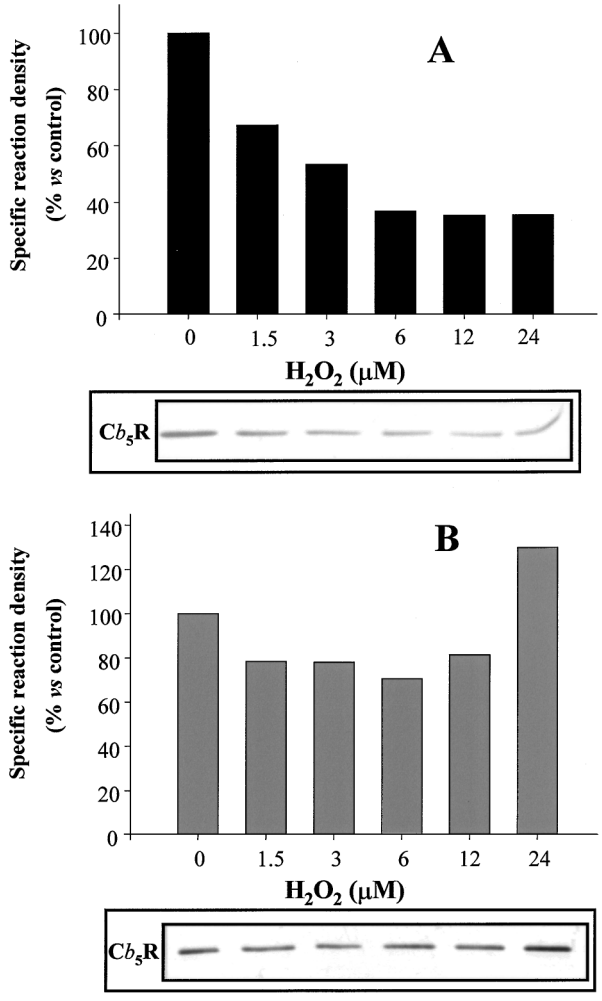

Fig. 3. Effect of exogenous H_2O_2 and cell density on cytochrome b_5 reductase polypeptide levels. Sparse (A) and confluent (B) HeLa cells were treated with sublethal concentrations of H_2O_2 for 13 h. Cells were then detached and homogenized for isolation of membrane fractions and cytochrome b_5 reductase quantification as described in Fig. 2. Increase values were relative to the amount of enzyme measured in membranes from sparse or confluent cells not treated with H_2O_2. Results and immunoblots presented are representative of three independent experiments.

reduced slightly the expression of Cytb_5R. Surprisingly, the highest noncytotoxic concentration of H_2O_2 (24 μM) produced a significant increase in the levels of Cytb_5R polypeptide (Fig. 3(B)).

To further prove that endogenous H_2O_2 is a factor that regulates Cytb_5R expression, we tested the effect of pyruvate, a well-characterized H_2O_2-scavenger (Giandomenico *et al.*, 1997). Addition of 10 mM pyruvate to cell cultures did not affect the HeLa cell growth rate with respect to cultures grown in its absence (Bello *et al.*, 2001). Unlike the effect on NQO1 activity, which was decreased by pyruvate treatment (Bello *et al.*, 2001), addition of 10 mM pyruvate resulted in an increase of Cytb_5R polypeptide. This effect was particularly evident in sparse cultures, where Cytb_5R levels in the presence of pyruvate were doubled compared to levels in its absence. Confluent cultures were less sensitive to scavenging of endogenous H_2O_2, but a 25% increase in Cytb_5R levels was still observed in the presence of 10 mM pyruvate (Fig. 4(A)).

In another series of experiment, endogenous ROS were generated by treating confluent HeLa cells with TBHQ (24 h, final concentration 60 μM), a compound which undergoes redox cycling by cellular quinone reductases, producing superoxide anion, hydrogen peroxide, and hydroxyl radicals (Kahl *et al.*, 1989). Addition of this quinone to culture media reduced about 66% the levels of Cytb_5R polypeptide found in confluent HeLa cells (Fig. 4(B)).

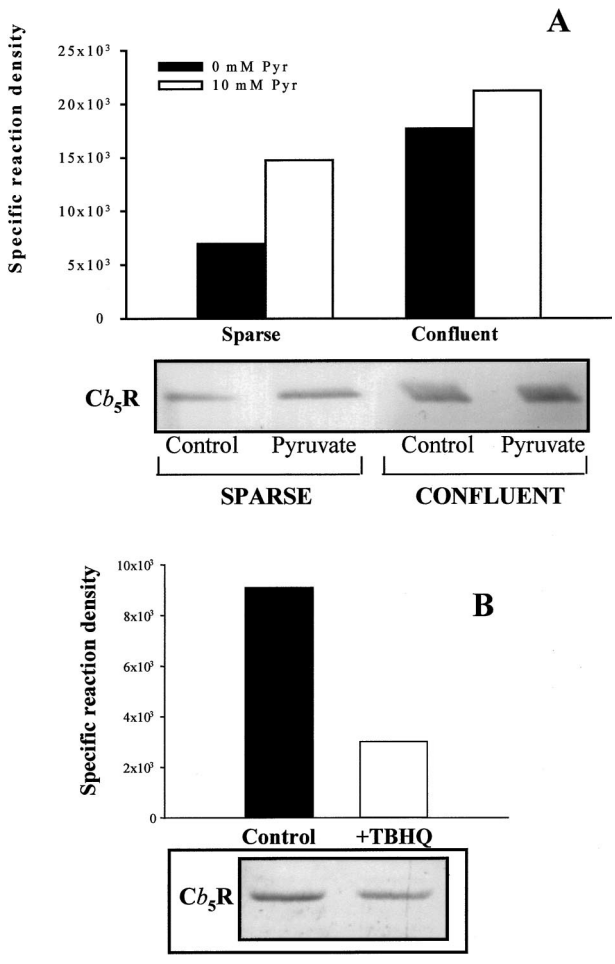

Fig. 4. Endogenous generation of reactive oxygen species and cytochrome b_5 reductase expression. (A) The effect of H_2O_2 scavenging with pyruvate. Cells were grown to the corresponding densities and then detached from culture plates for obtaining a membrane fraction as described under Material and Methods. Cultures were carried out either in the absence (closed bars), or in the presence (open bars) of 10 mM pyruvate for scavenging of endogenous H_2O_2. Data and immunoblot shown are representative of three independent experiments. (B) Effect of the redox cycling compound TBHQ on cytochrome b_5 reductase polypeptide levels in confluent HeLa cells. Cultures were treated for 24 h with 60 μM TBHQ in serum-free MEM. After tratment, cells were detached and homogenized for analysis of cytochrome b_5 reductase (see Fig. 2 legend). Stocks of TBHQ (60 mM) were made in ethanol, and a same volume of vehicle was added to controls without any noticeable effect on the levels of cytochrome b_5 reductase. Results and immunoblot presented are representative of two independent experiments.

Levels of ROS in Cultured HeLa Cells as a Function of Cell Density

Data reported earlier support that endogenous ROS could influence expression levels of the Cytb_5R in HeLa cells. It has been reported that H_2O_2 levels are not static but decline progressively with growth in BHK-21 fibroblast cells (Burdon *et al.*, 1995), and this density-associated decrease of ROS has been related to the density-dependent inhibition of growth in normal adherent cells (Pani *et al.*, 2000). However, no information is available to date about putative changes is ROS levels in HeLa cells, an adenocarcinoma cell line, as a function of density. Thus, we determined levels of ROS in both sparse and dense cultures of HeLa cells. Steady-state levels of intracellular peroxide and superoxide were significantly lower in confluent HeLa cells than in sparse cultures, as shown by flow cytometry studies using the probes DCFH-DA and Het for detection of peroxide and superoxide respectively (Fig. 5). Since we have previously demonstrated that H_2O_2 addition decreased Cytb_5R polypeptide in HeLa cells (see above), these results support that a decrease in endogenous ROS at high cellular density could be involved in the increase of Cytb_5R polypeptide observed under these conditions.

Cytochrome b_5 Reductase Levels in HL-60 Cells Adapted to H_2O_2

According to results presented in this paper, levels of Cytb_5R polypeptide decrease in cells submitted to acute oxidative stress, such as that caused by H_2O_2 addition. On the other hand, it has been reported that chronic oxidative

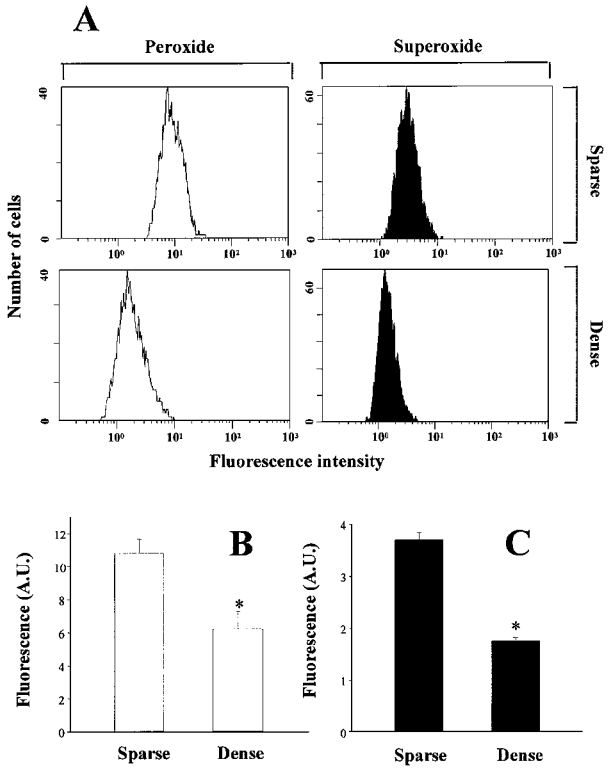

Fig. 5. Measurement of reactive oxygen species levels in sparse and dense cultures of HeLa cells. Cells were grown in MEM supplemented with 10% serum the corresponding densities (sparse: 8000 cells/cm^2; dense: 90,000 cells/cm^2) and incubated with 2′,7′-dichlorodihydrofluorescein diacetate and hydroethidine as probes for peroxide and superoxide respectively. After incubation, cells were detached and analyzed by flow cytometry (A). Results of quantification (in arbitrary units) of fluorescence mean values for each condition are presented in panels B (peroxide) and C (superoxide). Data are mean ± SD of two independent determinations.

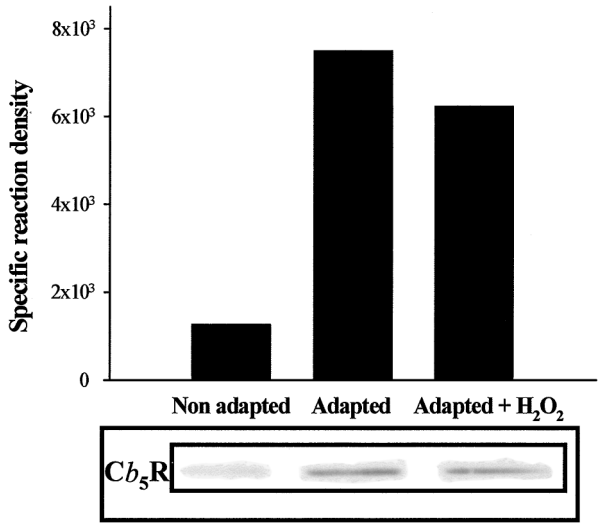

Fig. 6. Quantification of cytochrome b_5 reductase polypeptide in a HL-60-derived cell line adapted to H_2O_2. HL-60 cells were adapted to 100 μM H_2O_2 as described under Material and Methods. Membranes were isolated form parental HL-60 cells and H_2O_2-adapted cells grown in the absence of exogenous H_2O_2, and H_2O_2-adapted cells 13 h after addition of 100 μM H_2O_2. After electrophoresis (about 30 μg/lane), and western blotting, cytochrome b_5 reductase polypeptide was quantified as described in Fig. 2. Results and immunoblot presented are representative of three independent experiments.

stress, such as that provoked in laboratory animals by a vitamin E- and selenium-deficient diet, induces an increase in Cytb_5R (Navarro *et al.*, 1998). To test whether or not, prolongued exposure to H_2O_2 also increased levels of Cytb_5R, as a general response to chronic oxidative stress, we measured Cytb_5R polypeptide in membranes obtained from an HL-60-derived cell line adapted to H_2O_2. This line was obtained basically according to the procedure of Kasugai and Yamada (1992). These cells can be cultured in the presence of 100 μM H_2O_2, a concentration that is fully cytotoxic for the parental line, without any noticeable loss of viability. When cultured under standard conditions (i.e. in the absence of added H_2O_2), levels of Cytb_5R in membranes obtained from H_2O_2-adapted cells were six times higher than in parental HL-60 cells (Fig. 6). However, whereas adaptation of HL-60 cells to H_2O_2 resulted in an increase of steady-state levels of Cytb_5R polypeptide, addition of 100 μM H_2O_2 to these cells produced a short-term decrease in expression levels (Fig. 6), as previously found for HeLa cells.

DISCUSSION

The Cytb_5R is located on the cytosolic side of endomembranes and the plasma membrane (Borgese and Pietrini, 1986; Remacle, 1980; Villalba *et al.*, 1995). Besides its participation in different lipid biosyntethic pathways (Hildebrandt and Estabrook, 1971; Keyes and Cinti, 1980; Oshino *et al.*, 1971; Reddy *et al.*, 1977), and in the reduction of methemoglobin and cytochrome P_{450} (Hildebrandt and Estabrook, 1971; Hultquist and Passon, 1971), this enzyme can also reduce a variety of quinones by a one-electron mechanism to generate the corresponding semiquinones and hydroquinones in the absence of cytochrome b_5 (Nakamura and Hayashi, 1994). In this way, it has been reported that the Cytb_5R participates in the regeneration of ubiquinol and α-tocopherol (Constantinescu *et al.*, 1993, 1994; Navarro *et al.*, 1995; Villalba *et al.*, 1995, 1997). Also, an important role for Cytb_5R in the reduction of ascorbate-free radical to regenerate and maintain ascorbate in cells has been recognized (Lee *et al.*, 2001; Shirabe *et al.*, 1995; Villalba *et al.*, 1995). In sum, current evidence supports that the Cytb_5R is a member of the enzymatic machinery involved in antioxidant protection and in the control of the intracellular redox state.

Both oxidants and antioxidants are now recognized as important regulators of cell growth (Palmer and Paulson, 1997; Suzuki *et al.*, 1997). For instance, ROS are involved in both the initiation and promotion of carcinogenesis, whereas ROS scavengers are able to inhibit both stages. Moreover, altered levels of antioxidant enzymes have been observed during carcinogenesis or in tumors. When compared to their corresponding normal cell counterparts, tumor cells are low in Mn superoxide dismutase, Cu/Zn superoxide dismutase, and catalase, but glutathione-S-transferase, glucose-6-phosphatase and NOQ1 are increased in tumor cells, and Cytb_5R levels keep unaltered (Marín *et al.*, 1997; Sato *et al.*, 1992; Sun, 1990).

It is clear that there is not a common pattern in the regulation of antioxidant enzymes expression. Recently we have reported that NQO1 expression is dependent on the cell density in HeLa cells and can be upregulated by H_2O_2 (Bello *et al.*, 2001). We have used this cellular model for studying whether or not expression of the Cytb_5R, as another important antioxidant enzyme but generally considered as a constitutive housekeeping enzyme (Zenno *et al.*, 1990), is also affected by oxidative stress and the growth state of cells. For these studies, we determined the amount of enzyme present in cellular membranes using a specific antibody (Navarro *et al.*, 1995), since enzyme activity data do not correlate precisely with the presence of Cytb_5R (Borgese and Pietrini, 1986).

We have shown that levels of Cytb_5R polypeptide are significantly increased in confluent adenocarcinoma HeLa cells compared to sparse cultures. It has been shown that levels of antioxidant enzymes are generally modulated by cell density. Levels of other antioxidant enzymes also increase with growth. Catalase expression was higher in confluent hepatocytes than in earlier cultured cells (Röhrdanz and Kahl, 1998), and also in confluent HeLa cells compared to sparse cultures (Bello *et al.*, 2001). An increment in NQO1 expression has been also observed in confluent cultures of HeLa cells (Bello *et al.*, 2001; Collin *et al.*, 2001; Schlager *et al.*, 1993). Our results are also in accordance with those of Doostdar *et al.* (1990), who showed an increase of Cytb_5R expression in HepG2 hepatoma cells as the rate of cell growth slowed down. However, whereas the major increase of Cytb_5R activity in HepG2 cells occurs at confluency (Doostdar *et al.*, 1990), expression of Cytb_5R in HeLa cells already increased three fold during logarithmic growth phase, and reaching the confluency state did not result in any additional increment of the enzyme (see Fig 3). These results may indicate that, rather than cell-to-cell contacts, some diffusible factor could be involved in the observed increase of Cytb_5R. As shown for other proteins (Bello *et al.*, 2001; Garrido *et al.*, 1977), the demostration that the Cytb_5R is elevated in confluent adenocarcinoma cells is relevant to the area of bioreductive drug metabolism, because plateau phase cultures of tumor cells are valuable models that mimick many characteristics of the tumor microenvironment, such as reduced pH, poor nutrient status, low cell proliferation rates, and high catabolite concentrations (Phillips *et al.*, 1994; Phillips and Clayton, 1997).

Although all antioxidant enzymes protect the cell against ROS, the mechanism responsible for regulating their expression and the actual role of ROS themselves in this regulation can differ. H_2O_2 is constitutively generated by tumor cells (Burdon and Gill, 1993), and cells also alter their expression pattern of antioxidant enzymes in response to H_2O_2. Levels of some enzymes, such as catalase and Mn SOD, were increased, whereas Cu/Zn SOD was unaltered (Röhrdanz and Kahl, 1998). As we report in this paper for first time, exogenous H_2O_2 downregulates Cytb_5R polypeptide levels in HeLa cells, in both sparse and confluent cultures of HeLa cells, although sparse cells were much more sensitive to H_2O_2. However, enzymes involved in cellular antioxidant defense are generally upregulated by oxidative stress (Duthie and Collins, 1997; Kasugai and Yamada, 1992; Pinkus *et al.*, 1996; Röhrdanz and Kahl, 1998).

Several lines of evidence have allowed us to suggest that endogenous H_2O_2 is also involved in the regulation of Cytb_5R expression. First, treatment with pyruvate to scavenge endogenous H_2O_2 (Giandomenico *et al.*, 1997) resulted in a dramatic increase (about 200%) of Cytb_5R enzyme expression in sparse HeLa cells, reaching values nearly to those found in confluent cultures (Fig. 2), and a 25% increase was still observed for confluent HeLa cells treated with pyruvate compared to control cells. The higher increment of Cytb_5R polypeptide levels found for sparse HeLa cells cultured in presence of pyruvate could be explained by the removal of ROS generated during the early logarithmic phase of growth. Accordingly, steady-state levels of intracellular peroxide and superoxide were significantly lower in confluent HeLa cells than in sparse cultures, as shown by flow cytometry studies (see Fig. 6). Second, treatment of HeLa cells with TBHQ, as a source of endogenous ROS (including H_2O_2) derived from redox cycling of this compound (Pinkus *et al.*, 1996), resulted in a significant decrease of Cytb_5R polypeptide in confluent HeLa cells that was even stronger than the effect of H_2O_2 itself. In more complex systems, it has been shown that Cytb_5R activity is also modulated by the redox state of the organism. Repeated administration of the hypothyroidism-inducer propylthiouracil increases

selectively Cytb_5R, but not NADPH cytochrome P-450 reductase activity in rat microsomes (Kariya *et al.*, 1984). Interestingly, propylthiouracil inhibits H_2O_2 production by neutrophils or the activity of H_2O_2 in vitro (Imamura *et al.*, 1986).

Overall, our results suggest a redox regulation of Cytb_5R expression by H_2O_2. The promoter of human gene encoding Cytb_5R posseses five potential GC box sequences representing potential binding sites for the transcription factor SP1 (Toyoda *et al.*, 1995), which account for constitutive expression of Cytb_5R (Zenno *et al.*, 1990). DNA-binding efficiency of SP1 is affected by redox changes. Purified SP1 treated with H_2O_2 loses its ability to bind to its *cis* element, and the DNA-binding efficiency is fully restored after incubation with the antioxidant dithiothreitol (Ammendola *et al.*, 1994; Schäfer *et al.*, 1996). Futhermore, DNA-binding efficiency also depends on the DNA base damage by ROS, since oxidation of deoxiguanine residues at consensus binding sequences is sufficient to inhibit SP1 binding (Ghosh and Mirchell, 1999). In this way, it has been demonstrated that exogenous H_2O_2 produces a 60% inhibition in the expression of glycolytic enzymes by reversible oxidative inactivation of SP1 DNA binding in rat thymocytes (Hamm-Künzelmann *et al.*, 1997). The stronger inhibition of Cytb_5R expression by TBHQ than by exogenous H_2O_2 itself could be due to a direct damage of DNA caused by TBHQ metabolism (Dobo and Eastmond, 1994).

Inactivation of SP1 binding to promoter sequences of the Cytb_5R gene by oxidative stress fully agrees with our results showing a decrease of Cytb_5R by treatment with H_2O_2 and redox cycling of quinones, and an increase induced by H_2O_2 scavenging. Cell-density-regulated expression of Cytb_5R in HeLa cells could be consequence of a more reductant cellular redox state in confluent cells. Accordingly, we show in this paper that levels of ROS are significantly decreased in confluent cells. We have previously demonstrated that NQO1, a protective enzyme against redox damage, increases dramatically as HeLa cells reach confluency, and that catalase, a major cellular enzyme involved in the metabolism of H_2O_2, is also significantly increased in confluent HeLa cells (Bello *et al.*, 2001). Increases of other antioxidant enzymes (Oberley *et al.*, 1995) and decreases of ROS levels (Burdon *et al.*, 1995; Pani *et al.*, 2000) have been also described for confluent cultures of other cell lines. It is very likely that increments of antioxidant enzymes in confluent HeLa cells are related to the decrease of ROS. In turn, a decrease of ROS in confluent HeLa cells could explain both cell-density-related increase of the Cytb_5R, and a less effect of H_2O_2 scavenging with pyruvate. In addition to another enzymes which increase with cell density, an increment in Cytb_5R itself could also increase cell protection against oxidative stress because overexpression of mitochondrial Cytb_5R in yeasts makes the cells more resistant against H_2O_2 cytotoxicity (Lee *et al.*, 2001).

On the other hand, recent reports have documented that HeLa and human prostate carcinoma cells in dense cultures are subjected to mild hypoxic conditions (Kaluz *et al.*, 2002; Sheta *et al.*, 2001). In this way, pO_2 measured in equilibrated medium was 16% (116 mmHg), whereas pO_2 close to the surface of plated cells was 13% (96 mmHg) for sparse, and 9% (70 mmHg) for dense human prostate carcinoma cells. Although these changes in pO_2 are relatively small and are not as low as those seen in experimentally induced hypoxia (0.1–1%), formation of this small gradient is an important component of cell-density-mediated transcriptional activation because elimination of the pO_2 gradient by gentle shaking prevented the activation of a reporter gene driven by hypoxia-response element (Sheta *et al.*, 2001). Mild hypoxic conditions in dense cultures might be also involved in the regulation of density-dependent expression of Cytb_5R shown here.

Another point that deserves consideration is the stimulation of Cytb_5R expression by the highest noncytotoxic concentration of H_2O_2 (24 μM) in confluent cells. The increase of Cytb_5R by oxidative stress after a certain thereshold level suggests the existence of additional mechanisms of Cytb_5R expression regulation. Promoter region of the human Cytb_5R has been recently studied (Leroux *et al.*, 2001), and novel regulatory motifs have been identified which include a putative biding site for NF-κB. DNA-binding of NF-κB is strongly activated by H_2O_2 in Hela cells (Meyer *et al.*, 1993), and this transcription factor is an important mediator of redox-responsive gene expression that participates in cell adaptation to H_2O_2 (Kim *et al.*, 2001; Zhou *et al.*, 2001). Accordingly, HL-60 cells adapted to H_2O_2 (this work) and liver membranes obtained from rats subjected to chronic oxidative stress by feeding with diets deficient in vitamin E and selenium (Navarro *et al.*, 1998) exhibit increased levels of Cytb_5R. Molecular mechanisms that could mediate this adaptative increase of Cytb_5R to oxidative stress are currently under investigation.

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