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Original Research Communication

β-Carotene and Cigarette Smoke Condensate Regulate Heme Oxygenase-1 and Its Repressor Factor Bach1: Relationship with Cell Growth

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ABSTRACT

It has been reported that β-carotene is able to increase lung cancer risk in chronic smokers, but the mechanism for this association remains unknown. This article reports the first evidence that β-carotene, combined with cigarette smoke condensate (TAR), regulates heme oxygenase-1 (HO-1) *via* its transcriptional factor Bach1 and modulates cell growth. Both immortalized rat fibroblasts (RAT-1) and human lung cancer cells (Mv1Lu) exposed to TAR (25 μg/ml), exhibited an initial (6 h) induction of HO-1, followed by a late (24 h) repression due to the activation of Bach1. Heme oxygenase-1 repression was much more consistent when TAR was administered in combination with β-carotene (1 μ*M*) for 24 h; at this concentration the carotenoid *per se* did not have any effect on HO-1. Interestingly, the HO-1 repression following TAR *plus* β-carotene treatment caused a resynchronization of RAT-1 cell-cycle with a significant increase in the S-phase, and this was probably due to the decreased intracellular levels of carbon monoxide and bilirubin, both of which have antiproliferative effects. The role of HO-1 repression in increasing cell growth was also confirmed in Mv1Lu cells by the "knock down" of the Bach1 gene, thus demonstrating as HO-1 repression is a conserved mechanism by which cells can react to oxidative stress. *Antioxid. Redox Signal.* 8, 1069–1080.

INTRODUCTION

SEVERAL CLINICAL TRIALS have shown that β-carotene, when administered as a supplement, is able to increase the risk of lung cancer in chronic smokers (1, 18). Similar results have been found in an animal model: β-carotene, at pharmacological, but not at physiological concentrations, increased cell proliferation and induced detrimental histopathological changes in the lungs of cigarette smoke-exposed ferrets (9, 38). Moreover, Perocco *et al*. (22), in a cell culture bioassay of carcinogenesis with BALB/c3T3 cells, reported an enhancement of benzo[a]pyrene-induced transformation in cells treated with β-carotene. Very recently, we observed that β-carotene can exert detrimental effects in lung, mammary, larynx, and colon cancer cells, as well as in immortalized fibroblasts exposed to cigarette smoke condensate (TAR), by causing a remarkable increase in free-radical generation and modifying molecular pathways involved in cell proliferation and apoptosis (21). Although cigarette TAR has been reported to contain high concentrations of stable radicals, including a quinone/semiquinone/hydroquinone system, as observed by electron paramagnetic resonance (EPR) (24), the molecular mechanism(s) involved in the potential procarcinogenic effect of β-carotene and TAR in combination is still under debate.

Recent data suggested a key role of heme oxygenase-1 (HO-1) in the regulation of cellular homeostasis and growth (3, 37). Heme oxygenase-1 is the redox-sensitive inducible isoform of HO and its induction is one of the earlier cellular responses to tissue damage being responsible for the rapid transformation of the pro-oxidant heme into CO and BR, two

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molecules with antiinflammatory and antiapoptotic characteristics (10, 37). Recent studies demonstrated that HO-1 can be repressed in human cells exposed to hypoxia, thermal stress, and γ-interferon treatment (14, 17, 34); interestingly, the HO-1 repression seemed to be rather peculiar for humans, because the same stimuli were able to induce HO-1 in rodent cells (27). The importance of HO-1 repression has been corroborated by the discovery of Bach1 as heme-regulated repressor for transcription of HO-1 gene (19). Bach1 is a transcription factor widely expressed in mouse and human tissues (5, 19) and Bach1-deficient mice have *HO-1* gene overexpressed in many tissues, including brain (29). Furthermore, Bach1 is induced in cultured human cells by the same stimuli that repress HO-1 expression (7). Therefore, Bach1 plays a pivotal role in heme metabolism because it serves as a sensor for heme and represses HO-1 gene transcription depending on the heme availability (27).

Previous studies have shown that the transcription of HO-1 gene is induced by oxidant stimuli including reactive oxygen species (ROS), nitric oxide (NO), bacterial lipopolysaccharide (LPS), and hemin (6, 11, 32, 33, 39). In particular, it has been recently reported that human monocytes and endothelial cells exhibited an increase in the HO-1 mRNA and protein as a consequence of cigarette smoke exposure (4, 36). In addition β-carotene, a known modulator of the intracellular redox status, is able to affect HO-1, although it is still unclear if the carotenoid increases or decreases this protein (15, 16, 35).

In the present work, we suggest that the detrimental effects of cigarette smoke combined with β-carotene can be related to changes in HO-1. Therefore, we evaluated in rat fibroblasts (RAT-1) and human lung carcinoma cells (Mv1Lu) the role of TAR and β-carotene, alone and/or in association, on HO-1 gene expression and protein, and the possible involvement of the repressor Bach1 in the mechanism leading to the HO-1 gene repression. Furthermore, we studied how the modulation of HO-1 can affect cell growth in both the cell lines.

MATERIALS AND METHODS

Cell culture

RAT-1 rat immortalized fibroblasts (American Type Culture Collection, Rockville, MD) were grown in MEM medium without antibiotics, supplemented with 10% fetal calf serum and 2 m*M* glutamine. Mv1Lu human lung carcinoma cells (American Type Culture Collection) were cultured in D-MEM medium without antibiotics, supplemented with 10% fetal calf serum (Flow, Irvine, UK) and 2 m*M* glutamine. Cells were maintained in log phase by seeding twice a week at the density of 3×10^5 cells/ml at 37°C under 5% CO_2/air atmosphere. TAR was delivered to the cells as dimethyl sulfoxide (DMSO) solutions. The amount of DMSO given to the cells was not >0.1% (v/v). The final concentration given to the cells was 25 μg/ml. Such a concentration was the maximum one that did not induce toxic effects in all the cells analyzed (21). β-Carotene (Fluka Chemika-bioChemika, Buchs, Switzerland) was delivered to the cells (10^6 cells/ml) using tetrahydrofuran (THF) as a solvent, containing 0.025% butylated hydroxytoluene (BHT) to avoid the formation of peroxides (21). The purity of β-carotene was verified to be 97% by HPLC. The stock solutions of β-carotene and α-tocopherol (Fluka Chemika-bioChemika) were prepared immediately before each experiment. From the stock solutions, aliquots of β-carotene and/or α-tocopherol were rapidly added to the culture medium to give the final concentrations indicated. The amount of THF added to the cells was not >0.1% (v/v). Control cultures received an amount of solvent (DMSO and/or THF) equal to that present in TAR and β-carotene-treated ones. No differences were found between cells treated with DMSO and/or THF and untreated cells in terms of cell number and viability. After the addition of β-carotene, the medium was not further replaced throughout the experiments. Carbon monoxide was delivered to cells using the CO-releasing molecule (CO-RM) tricarbonyldichlororuthenium (II) dimer (Sigma Aldrich, Milan, Italy) dissolved in DMSO at the stock concentration of 10 m*M* (13). Bilirubin (Sigma Aldrich) and Sn-protoporphyrin-IX (Sn-PP-IX) (Biomol International/Affiniti, Devon, UK) were dissolved in NaOH 0.1 *M* at the stock concentration of 10 m*M*. Working solutions of CO-RM and BR have been prepared, diluting these substances directly in the cell culture medium. Control cells received an amount of DMSO or NaOH equal to that present in CO-RM or BR treated ones, respectively. Both the CO-RM and BR are light sensible and therefore the experiments were carried out in dim light. Small interfering RNA (siRNA) for Bach1 and related products were purchased by Santa Cruz Biotechnology (DBA, Milan, Italy). Mv1Lu cells were plated the day before transfections and grown to 60%–70% confluence in 12-well plates. The cells were transfected with 100 n*M* Bach1 siRNA using the transfection reagent as described by the manufacturer. Transfected cells were grown for 24 h in normal growth medium and then treated as indicated in the "Results" section. Control experiments were performed transfecting Mv1Lu cells with a control siRNA provided by Santa Cruz Biotechnology that consists of a scrambled sequence that do not lead to the specific degradation of any cellular message.

At the appropriate time-points, cells were harvested and quadruplicate hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

TAR preparation

The particulate phase of cigarette smoke condensate (TAR) was provided by BAT Italia (British American Tobacco, Rome, Italy). It was obtained by mechanically smoking cigarettes, using a smoking machine (Cerulean, ASM 516 model). Cigarettes were smoked using the puff profile (one 35-ml puff/min) to a butt length of 2.3 cm, as indicated by BAT protocol. The experimental conditions during smoking were: 22°C temperature, 60% humidity. The TAR from 20 cigarettes was trapped on filters and then extracted with deionized water. The aqueous solutions were filtered through a Whatman (0.2 μm) filter and dried in vacuum at room temperature. The dried cigarette total particulate matter was redissolved in DMSO and stored at –20°C.

Protein assay

Proteins were measured using the Bio-Rad protein Assay following manufacturer's instructions (Bio-Rad Italy, Milan, Italy).

Western blot analysis

Cells (1×10^7) were harvested, washed once with ice-cold phosphate buffered saline, and lysed as described in Ref. 21. Cell lysates were then cleared by centrifugation at 10,000 *g* for 10 min at 4°C. Nuclear extracts of RAT-1 and Mv1Lu cells were used for the detection of Bach1 protein. Equal amounts of proteins (30 and 5 μg for HO-1 and Bach1, respectively) were subjected to SDS–PAGE electrophoresis, transferred on a nitrocellulose membrane- and the nonspecific binding of antibodies blocked by 3% nonfat dried milk in PBS. Immunodetection of HO-1 and Bach1 proteins were carried out using a rabbit polyclonal antibody (1:1,000, Stressgen, Victoria, Canada) and anti-Bach1 (1:1,000) (30), respectively. The blots were washed and exposed to horseradish peroxidase-labelled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR)

HO-1 mRNA expression was evaluated by reverse transcription polymerase chain reaction (RT–PCR). Total RNA was extracted from 10^7 RAT-1 fibroblasts by Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. Total RNA (1.25 μg) was then reverse transcribed to cDNA by Moloney-murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen). The reaction was performed in a final volume of 20 μl, using 200 ng of random primers (Invitrogen) and 200 U of M-MLV RT, 0.8 m*M* deoxynucleotide triphosphates (dNTPs, Promega, Madison, WI), 20 U of recombinant RNasin ribonuclease inhibitor (Promega), 0.05 *M* dithiothreitol in the buffer provided by the M-MLV RT manufacturer. The reaction was carried out for 60 min at 37°C. cDNA samples were stored at –20°C until PCR amplification.

Semiquantitative PCR was used to measure HO-1 mRNA, with β-actin mRNA as the internal control, as follows. From preliminary experiments, cDNA dilutions of 1:20 and 1:5,000 for HO-1 and β-actin, respectively, were chosen that were in the exponential phase of the amplifications, and used in all subsequent PCR. For each sample, HO-1 and β-actin cDNAs were amplified in separate tubes. PCR was performed in a final volume of 50 μl, using 5 μl of 1:2.5 diluted first strand cDNA sample and 20 pmol of each primer (Eurobio, Courtaboeuf Cedex 8, France), 0.16 m*M* dNTPs, and 1.25 U of *Taq* polymerase (Promega), in a buffer provided by the *Taq* polymerase manufacturer. After an initial denaturation at 94°C for 2 min, 30 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed. Preliminary experiments showed that 30 cycles of PCR were within the exponential phase of HO-1 and β-actin cDNA amplifications. After PCR cycles, reactions were further incubated at 72°C for 10 min and then chilled at 4°C. Sequences of oligonucleotide primers specific for HO-1 and β-actin were chosen according to Sammut *et al*. (26) and are shown in Table 1. Primers have been designed to span one intron within the genes to check any possible genomic contamination of cDNA samples. PCR products were then loaded on a 1.2% agarose gel and electrophoresed in 1× Tris-borate-EDTA (TBE) buffer. Bands were stained with ethidium bromide (0.5 μg/ml). The optical density (IOD) of each band was quantitated by a CCD video camera-based system (ImageMaster VDS gel documentation system, Amersham Biosciences) using the dedicated image analysis software. HO-1 gene expression levels were expressed as the ratios of IOD of HO-1 and β-actin bands.

Cell cycle analysis

Cell cycle stage was analyzed by flow cytometry. Aliquots of 10^6 cells were harvested by centrifugation, washed in PBS, fixed with ice-cold ethanol, and treated with 1 mg/ml RNase for 30 min. Propidium iodide was added to a final concentration of 50 mg/ml. Data were collected, stored, and analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDE PRIMERS USED FOR RT–PCR

Gene amplified	*Sequence (5′ → 3′)*	*Orientation*	*Location*	*PCR product size (bp)*
HO-1	CTTTCAGAAGGGTCAGGTGTCCA	Sense	Exon 2, nt # 3,523–3,545*	320
	CTGAGAGGTCACCCAGGTAGCGG	Antisense	Exon 3, nt # 4,668–4,646	
β-Actin	CGTGGGCCGCCCTAGGCACCA	Sense	Exon 2, nt # 1,345–1,365†	246
	CGGTTGGCCTTAGGGTTCAGAGGGG	Antisense	Exon 3, nt # 1,677–1,653	

*GenBank accession number J02722.
†GenBank accession number V01217.

Immunocytochemistry

Cytospins of RAT-1 fibroblasts prepared with Shandon Cytospin 3 (Shandon, Keshire, UK) were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with cold (−20°C) methanol for 10 min. Cells were then washed with PBS and incubated for 1 h at room temperature with PBS containing anti-Bach1 antibody (30). Mouse preimmune Igs were used as negative control. Endogenous biotin sites were blocked by sequential incubations with avidin–biotin solutions (Blocking kit; VectorLab, Burlingame, CA). Hydrogen peroxide, normal goat blocking serum, biotinylated Igs avidin–biotin complexes, and DAB substrate solutions (ABC Elite Detection System; VectorLab) were used according to the manufacturer's instructions. For each slide four randomly selected microscopic fields were observed and at least 100 cells/field were evaluated.

Statistical analysis

Values were presented as means ± SEM of n experiments. Multifactorial two-way analysis of variance (ANOVA) was adopted to assess any differences among the treatments and the times. When significant values were found ($p < 0.05$), *post hoc* comparisons of means were made using the Tukey's Honestly Significant Differences test.

One-way ANOVA was used to determine differences among concentrations. When significant values were found

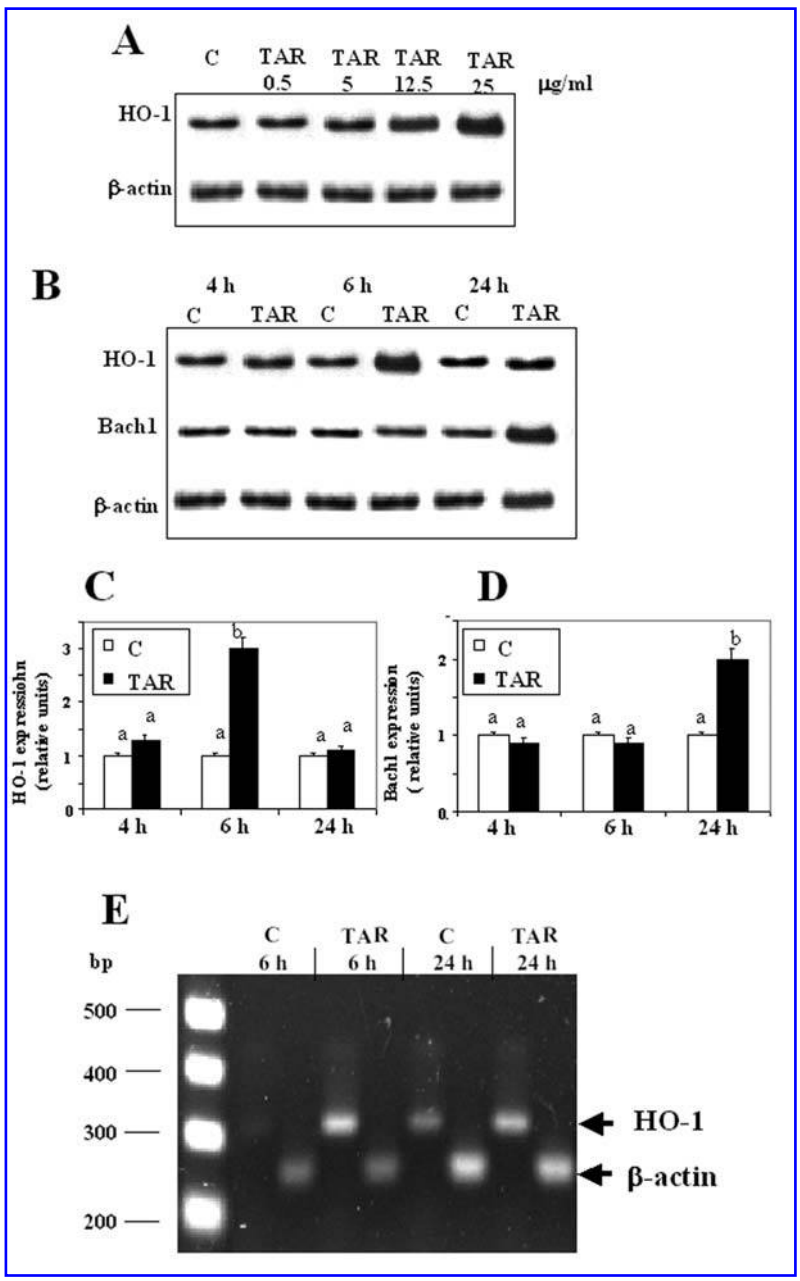

FIG. 1. Effects of TAR on HO-1 and Bach1 gene expression and protein. (**A**) RAT-1 fibroblasts were treated with TAR (0.5–25 µg/ml) for 6 h and the cellular extracts were analyzed by immunoblot using an anti-HO-1 antibody. (**B**) RAT-1 fibroblasts were treated with TAR (25 µg/ml) and the cells collected after 4, 6, and 24 h. Cellular extracts were then analyzed by immunoblot using anti-HO-1 and anti-Bach1 antibodies. (**C**) and (**D**) densitometric evaluation of the immunoblots shown in (**B**). (**E**) RAT-1 fibroblasts were treated with TAR (25 µg/ml) for 6 and 24 h. At these time points the cells were collected, the total RNA extracted, and the RT–PCR performed as described in Materials and Methods. In (**C**) and (**D**), data are expressed as relative units, mean ± SEM, $n = 3$; the treatment/time interaction was significant ($p < 0.05$) two-way ANOVA. Values not sharing the same superscripts were significantly different (**C**: $p < 0.001$; **D**: $p < 0.002$, Tukey's test). A representative experiment is shown in (**A**), (**B**), and (**E**). C, Control.

($p < 0.05$), *post hoc* comparisons of means were made using Fisher's Test. Differences were analyzed using Minitab Software (Minitab Inc., State College, PA).

RESULTS

Effects of TAR on HO-1 and Bach1

TAR, administered to RAT-1 fibroblasts in the range 0.5–25 μg/ml for 6 h, increased HO-1 protein in a dose dependent manner (Fig. 1A). Time-course studies using TAR 25 μg/ml showed that HO-1 levels changed over time with a slight increase at 4 h, which became strong at 6 h, followed by a decrease at 24 h (Figs. 1B and C). Such a behavior of TAR on HO-1 protein paralleled the effects of this compound on HO-1 gene expression, as evidenced by RT–PCR (Fig. 1E). In fact TAR 25 μg/ml significantly increased HO-1 gene expression after 6 h of incubation, whereas after 24 h the gene expression decreased (HO-1/β-actin ratio: 8.0 ± 2.0 and 2.0 ± 0.5 folds versus control, respectively, $p < 0.01$) suggesting that the modifications induced by TAR occurred at a transcriptional level.

The decrease of HO-1 protein levels *vis-à-vis* with the repression of its gene expression seen after 24 h of incubation, prompted us to study the possible involvement of the transcription repressor Bach1. As shown in Fig. 1, panels B and D, TAR 25 μg/ml did not change Bach1 levels at 4 and 6 h, but significantly induced this protein after 24 h of incubation.

Effects of β-carotene on HO-1 and Bach1

Exposure of RAT-1 fibroblasts for 24 h to β-carotene at the doses of 2–5 μ*M* resulted in a dose-dependent decrease of HO-1 protein, while β-carotene 1 μ*M* did not have any significant effect (Figs. 2A and B). Similar results were obtained studying the HO-1 gene expression that was strongly repressed only by β-carotene 5 μ*M* (HO-1/β-actin ratio: control 0.40 ± 0.05 and β-carotene 5 μ*M* 0.23 ± 0.02, $p < 0.01$) (Fig. 2D).

Keeping in mind the results obtained with TAR, Bach1 levels were also evaluated in these experiments. Western Blot analysis demonstrated a significant increase of Bach1 in fibroblasts treated for 24 h with β-carotene 2–5 μ*M,* whereas the latter, at the concentration of 1 μ*M*, did not show any significant effect on the repressor protein levels (Figs. 2A and 2C).

Effects of the association of TAR and β-carotene on HO-1 and Bach1 in rat fibroblasts

When RAT-1 fibroblasts were treated for 4, 6, 12, and 24 h with TAR 25 μg/ml and β-carotene 1 μ*M* in association, the HO-1 protein levels changed over time with a significant increase after 4 h, followed by a decrease after 6 h of incubation (Figs. 3A and B). This progressive reduction of HO-1 was confirmed at later time points including 12 h (control 1.00 ± 0.06, TAR 2.00 ± 0.15, β-carotene 0.90 ± 0.07, TAR + β-carotene 1.20 ± 0.10 relative units, mean ± SEM, $n = 3$) and 24 h (Figs. 3A and B) Such an effect was accompanied by a parallel increase in Bach1 levels that became significant after 6 h (Figs. 3A and C) and further increased after 12 h (control 1.00 ± 0.04, TAR 1.40 ± 0.10, β-carotene 1.05 ± 0.05, TAR + β-carotene 3.00 ± 0.20 relative units, mean ± SEM, $n = 3$) and 24 h of incubation (Figs. 3A and C).

The HO-1 repression was also substantiated by RT–PCR, as shown in Fig. 3D. RAT-1 fibroblasts, after 24 h of incubation with TAR + β-carotene, have HO-1 gene expression comparable to the control levels, whereas TAR alone exhibited a twofold increase.

To verify the hypothesis that a prooxidant mechanism was involved in the regulation of the HO-1/Bach1 system, we exposed RAT-1 fibroblasts to increasing concentrations of α-tocopherol (1–25 μ*M*) in the presence of TAR alone (25 μg/ml) and in combination with β-carotene (1 μ*M*) for 24 h. We found that α-tocopherol inhibited Bach1 activation in a dose-dependent manner (data not shown); the antioxidant, at the concentration of 25 μ*M*, significantly inhibited Bach1 activation induced by TAR alone and in combination with β-carotene (75.0 ± 8.0% and 69.0 ± 5.0%, respectively, *versus* matched α-tocopherol-untreated cells, mean ± SEM, $n = 3$, $p < 0.01$).

Bach1 immunocytochemistry

The involvement of Bach1 in HO-1 gene repression in our model was also confirmed by immunocytochemical studies. In control cells, Bach1 immunostaining accumulated within the cytoplasmic region with clear nuclear exclusion; this finding is consistent with previous data showing that Bach1 accumulates in the cytosol if overexpressed in many mammalian cell lines (31), whereas, when the fibroblasts were treated with TAR 25 μg/ml for 24 h, Bach1 translocated within the nucleus consistently with its repressor activity on DNA. Interestingly, β-carotene 1 μ*M per se* was ineffective, but when given in association with TAR 25 μg/ml, it increased Bach1 nuclear accumulation at a greater extent than TAR alone (Fig. 4). Incidentally, β-carotene *per se* was able to induce Bach1 translocation from the cytoplasm into the nucleus but only at 5 μ*M* (data not shown).

Effects of TAR and β-carotene on the cell cycle of RAT-1 fibroblasts

Table 2 shows the cell cycle of RAT-1 fibroblasts treated with TAR 25 μg/ml and β-carotene 1 μ*M* for 24 h. In the absence of TAR and β-carotene, about half of the cells were in the G0/G1 phase and no significant differences were observed in cells treated with β-carotene alone. RAT-1 fibroblasts exposed to TAR showed a slight increase in the percentage of cells in G0/G1 phase, whereas when TAR and β-carotene were given in combination, the fibroblasts exhibited a significant reduction in the percentage of G0/G1 phase paralleled by an increase in the S phase. These data demonstrated that at 24 h, cells exposed to TAR + β-carotene recovered from the G0/G1 block and started to replicate.

Effects of carbon monoxide, bilirubin and Sn-protoporphyrin-IX on the cell growth of RAT-1 fibroblasts

One of the reasons why the cells repress HO-1 is to avoid the accumulation of BR and CO, which are toxic if produced

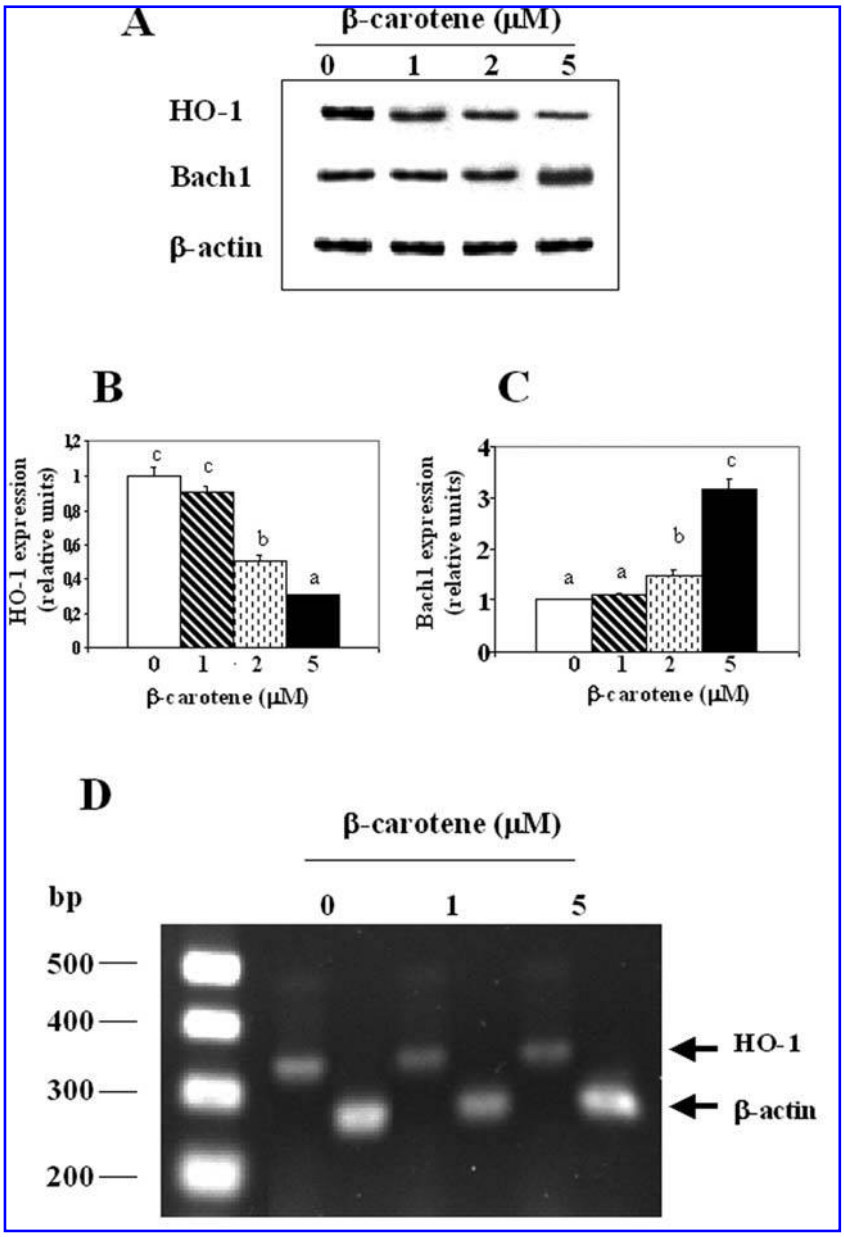

FIG. 2. Effects of β-carotene on HO-1 and Bach1 gene expression and protein. (**A**) RAT-1 fibroblasts were treated with β-carotene (0–5 μ*M*) for 24 h. Cellular extracts were then analyzed by immunoblot using anti-HO-1 and anti-Bach1 antibodies. (**B**) and (**C**) densitometric evaluation of the immunoblots shown in (**A**). (**D**) RAT-1 fibroblasts were treated with β-carotene (1 and 5 μ*M*) for 24 h. Cells were then collected, the total RNA extracted, and the RT–PCR performed as described in Materials and Methods. In (**B**) and (**C**), data are expressed as relative units, mean ± SEM, $n = 3$. Values not sharing the same superscripts were significantly different ($p < 0.05$) one-way ANOVA. A representative experiment is shown in (**A**) and (**D**).

in excess, as in the case of prolonged HO-1 induction (27). On this basis, we tested the hypothesis whether BR and CO could affect the growth of RAT-1 fibroblasts. Carbon monoxide was delivered to the cells by using the CO-RM tricarbonyldichlororuthenium (II) dimer as described in Materials and Methods.

As shown in Figs. 5A and B, BR or CO (0.1–10 μ*M*) dose-dependently reduced RAT-1 cell growth and this effect became significant at the concentration of 1 μ*M*. To exclude a confounding effect on cell viability due to the ruthenium contained in the CO-RM, control experiments using ruthenium (II) chloride were performed. Ruthenium chloride (0.1–10 μ*M*) did not have any significant effect on RAT-1 cell growth (data not shown). Because during heme catabolism CO and BR are formed in equimolecular amounts, we tested the effect of CO and BR in combination on RAT-1 cell growth. As shown in Fig. 5C, BR *plus* CO reduce RAT-1 cell growth at a greater extent than the single molecules alone.

The hypothesis that HO-1 activity is able to regulate the growth of RAT-1 fibroblasts was also strengthened by using the HO inhibitor Sn-protoporphyrin-IX (Sn-PP-IX). RAT-1 cells exposed to Sn-PP-IX (10 μ*M*) for 24 h showed a significant increase in cell growth (control: $750 \pm 30 \times 10^3$, Sn-PP-IX: $900 \pm 47 \times 10^3$, mean ± SEM, $n = 6$, $p < 0.05$).

Effects of the association of TAR and β-carotene on HO-1 and Bach1 in human lung cancer cells

To see whether HO-1 repression following β-carotene and TAR treatment could occur in humans, we studied this phenomenon also in human lung cancer cells. In fact, it is known

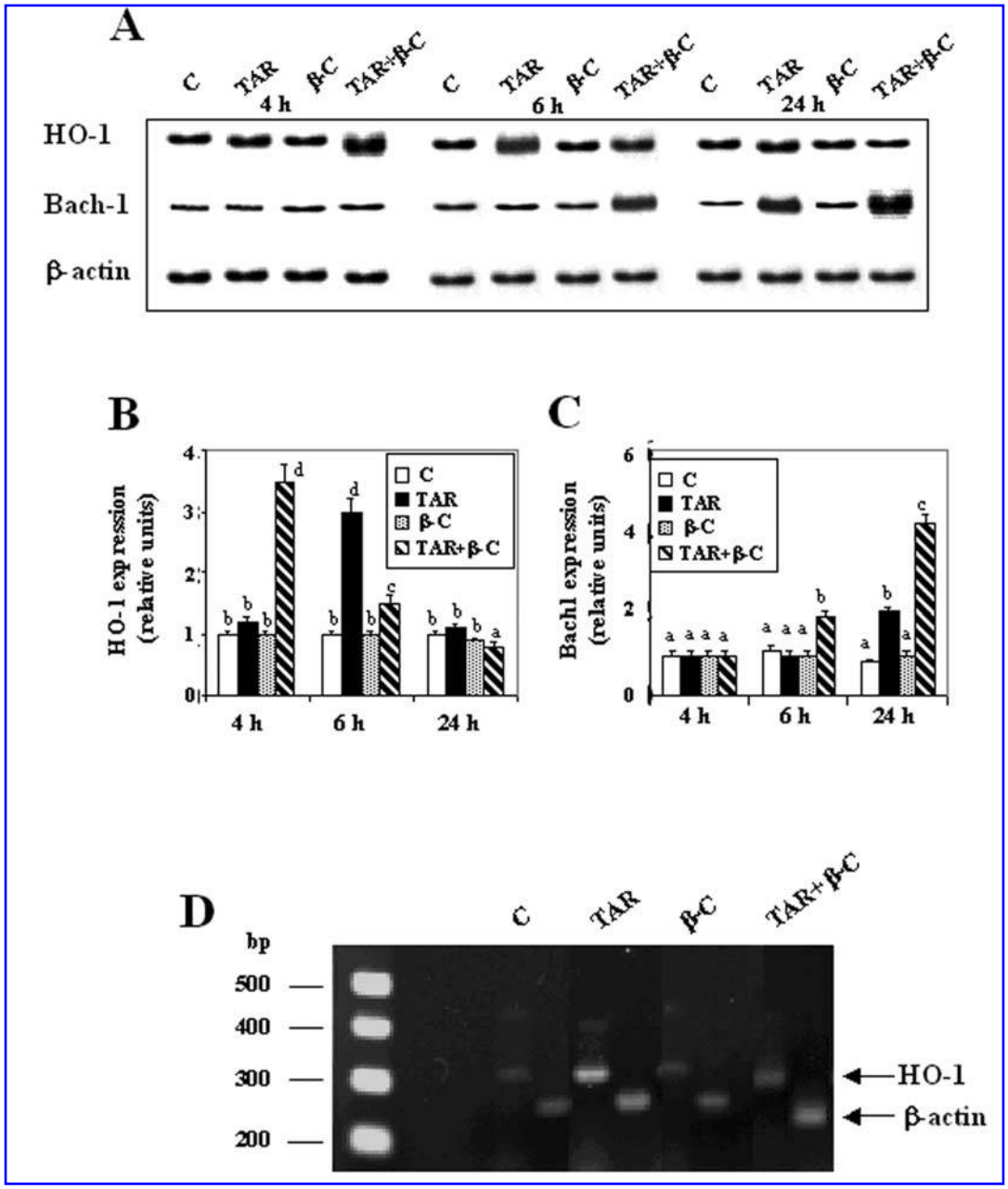

FIG. 3. Effects of TAR and β-carotene in combination on HO-1 and Bach1 gene expression and protein. (**A**) RAT-1 fibroblasts were treated with TAR (25 μg/ml) and β-carotene (1 μ*M*), alone and/or in association, for 4, 6, and 24 h. Cellular extracts were then analyzed by immunoblot using anti-HO-1 and anti-Bach1 antibodies. (**B**) and (**C**) densitometric evaluation of the immunoblots shown in (**A**). (**D**) RAT-1 fibroblasts were treated as in (**A**) for 24 h. Cells were then collected, the total RNA extracted, and the RT–PCR performed as described in Materials and Methods. In (**B**) and (**C**), data are expressed as relative units, mean ± SEM, $n = 3$; the treatment/time interaction was significant ($p < 0.05$), two-way ANOVA. Values not sharing the same superscripts were significantly different (**B**: $p < 0.001$; **C**: $p < 0.005$, Tukey's test). A representative experiment is shown in (**A**) and (**D**). c, control; β-c, β-carotene.

that the lung is one of the more sensitive organs to cigarette smoke damage.

Human Mv1Lu lung carcinoma cells treated with TAR 25 μg/ml alone and in association with β-carotene 1 μ*M* exhibited a marked increase in HO-1 protein after 6 h of incubation, followed by a late decrease at 24 h. Constantly, Bach1 protein levels increased after 24 h of incubation, the time point at which HO-1 was strongly decreased (Fig. 6A).

To examine the relevance of HO-1/Bach1 system in modulating the growth of Mv1Lu cells we decided to "knock-down" Bach1 by using Bach1 siRNA. After 24 h of transfec-

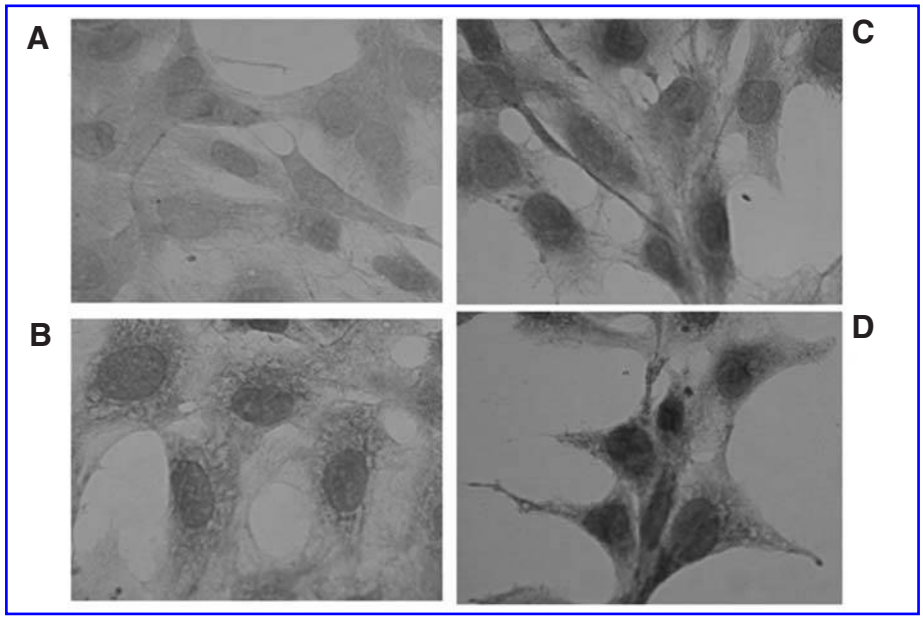

FIG. 4. Effects of TAR and β-carotene on Bach1 intracellular localization. RAT-1 fibroblasts were treated with TAR (25 μg/ml) and β-carotene (1 μ*M*) alone and in combination for 24 h. The experiment was performed in quintuplicate and selected fields are shown. (**A**) Control; (**B**) β-carotene; (**C**) TAR; (**D**) TAR *plus* β-carotene.

tion, the cells exhibited a significant decrease of both Bach1 gene expression (data not shown) and protein (Fig. 6B). As a consequence of Bach1 knock-down, HO-1 expression in Mv1Lu cells treated with TAR alone and in combination with β-carotene for 24 h was not repressed (Fig. 6C). Furthermore, as shown in Fig. 6D, Mv1Lu cells transfected with siRNA for Bach1 and treated for 24 h with TAR in combination with β-carotene exhibited a significant decrease in cell growth with respect to control cells, whereas TAR alone did not have any significant effect.

DISCUSSION

In this article, we suggest that the modulation of HO-1 can be a putative mechanism through which the association of β-carotene and cigarette smoke exerts the procarcinogenic effects reported in previous clinical trials and experimental studies (1, 18, 21). In particular we studied (a) HO-1 gene expression and protein in both RAT-1 fibroblasts; and (b) Mv1Lu lung carcinoma cells treated with β-carotene and exposed to TAR; as well as (c) the consequences of HO-1 stimulation/inhibition on cell growth. The rationale to include rat fibroblasts in this study is double: first because idiopathic pulmonary fibrosis is a very frequent cause or consequence of lung cancer and is often related to cigarette smoking (25), and second because we wanted to see whether TAR and β-carotene modulated differently HO-1 in rodent cells with respect to humans.

Previous studies have shown that cigarette smoke, which contains a great abundance of free radicals, is able to induce HO-1 mRNA and protein in human monocytes and endothelial cells, this effect being mediated by a 20 bp fragment located in the HO-1 promoter region and called cadmium-responsive element (CdRE); this fragment interacts with the Fos/Jun transcription factors that, in turn, strongly increase HO-1 expression (4, 36). Our results confirmed the early activation of HO-1 following TAR 25 μg/ml exposure either at the gene or protein level and provide evidence on the late involvement of the repressor transcription factor Bach1 as a negative regulator to avoid a prolonged induction of this enzyme. In fact, Bach1 levels were strongly increased after the exposure of RAT-1 and Mv1Lu cells to TAR for 24 h, the same time point at which the HO-1 gene expression and protein were very low.

TABLE 2. EFFECTS OF β-CAROTENE AND TAR ON CELL CYCLE DISTRIBUTION OF RAT-1 FIBROBLASTS

	Cell Cycle 24 h		
Treatment	*G0/G1*	*S (%)*	*G2/M*
Control	53.0 ± 2.0 †	25.9 ± 2.0*	21.1 ± 1.5
β-Carotene 1 μ*M*	51.4 ± 2.0†	26.1 ± 1.3*	22.5 ± 2.1
TAR 25 μg/ml	58.1 ± 2.2†	22.4 ± 1.1*	19.5 ± 1.9
TAR + β-Carotene	49.9 ± 1.8*	31.1 ± 1.1†	19.0 ± 2.1

Within a column, values not sharing the same superscript were significantly different ($p < 0.05$). The values were the means ± SEM of six experiments.

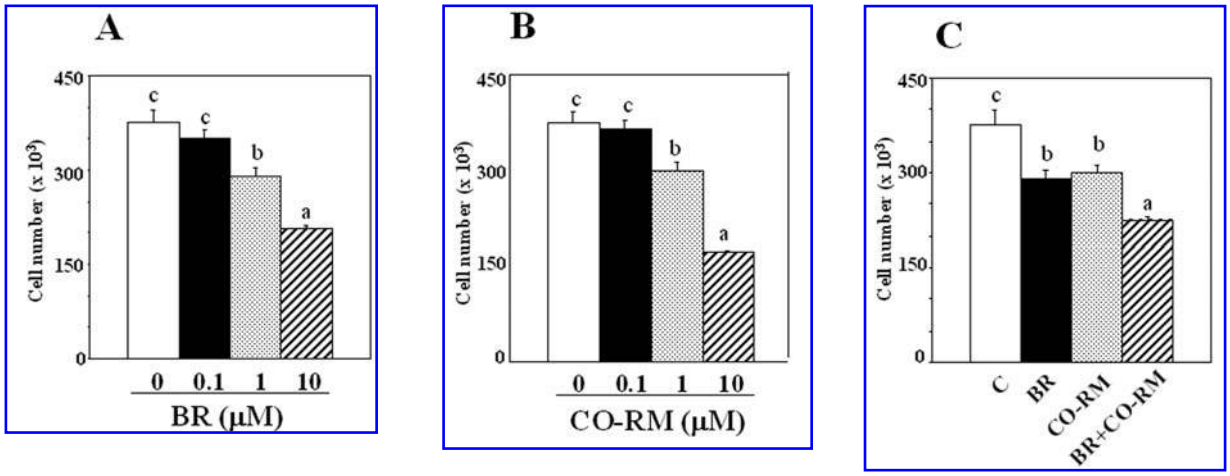

FIG. 5. Effects of BR and CO-RM on RAT-1 fibroblast cell growth. Cells were treated with BR (0–10 μ*M*) (**A**), CO-RM (0–10 μ*M*) (**B**), and with BR (1 μ*M*) and CO-RM (1 μ*M*) alone and in combination (**C**) for 24 h. Data are expressed as mean ± SEM, $n = 6$. Values not sharing the same superscripts were significantly different ($p < 0.05$), one-way ANOVA. BR, bilirubin; CO-RM, CO-releasing molecule.

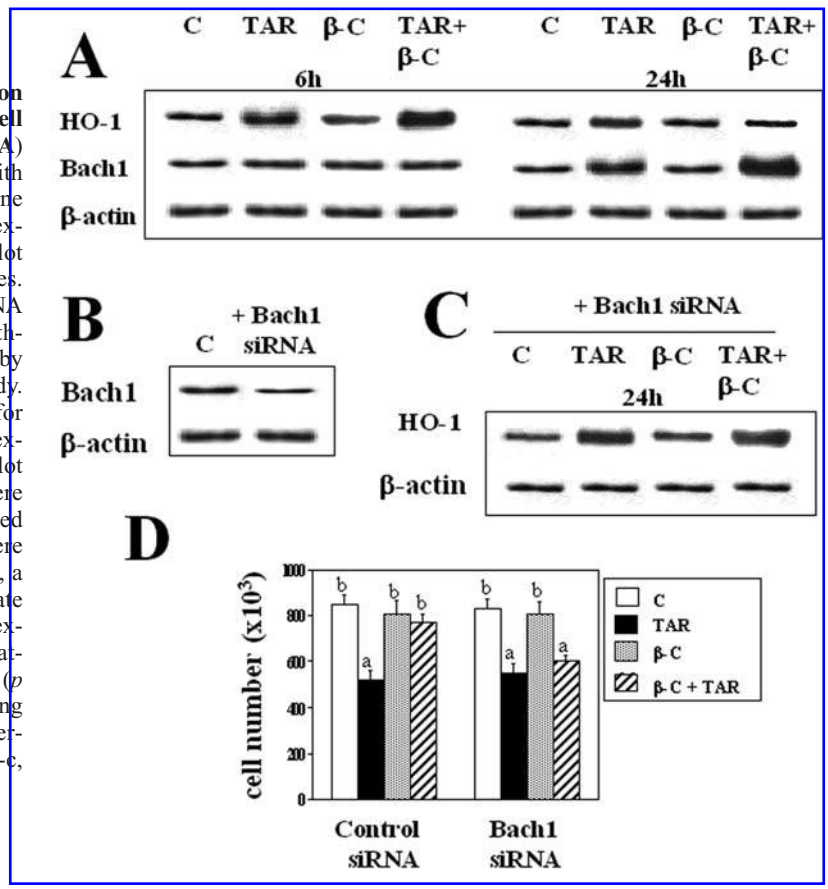

FIG. 6. Effects of TAR and β-carotene on HO-1 and Bach1 proteins and on cell growth in human lung cancer cells. (**A**) Mv1Lu lung carcinoma cells were treated with TAR (25 μg/ml) and β-carotene (1 μ*M*), alone and/or in association, for 24 h. Cellular extracts were then analyzed by immunoblot using anti-HO-1 and anti-Bach1 antibodies. (**B**) Mv1Lu cells were transfected with siRNA for Bach1 as described in Materials and Methods. Cellular extracts were then analyzed by immunoblot using an anti-Bach1 antibody. (**C**) Cells were transfected with siRNA for Bach1 and then treated as in (**A**), cellular extracts were then analyzed by immunoblot using an anti-HO-1 antibody. (**D**) Cells were transfected with siRNA for Bach1 and treated as in (**A**). At the end of incubation cells were collected and counted. In (**A**), (**B**), and (**C**), a representative experiment of three separate immunoblots is shown; in (**D**), data are expressed as mean ± SEM, $n = 3$. The treatment/treatment interaction was significant ($p < 0.05$), two-way ANOVA. Values not sharing the same superscript were significantly different ($p < 0.005$, Tukey's test). C, Control; β-c, β-carotene.

A similar modulation on HO-1 gene expression and protein was observed when RAT-1 fibroblasts were treated with β-carotene, which is known to act as an intracellular redox agent.

Previous studies demonstrated an overproduction of free radical species by high concentrations of β-carotene itself (≥ 5 μ*M*), whereas at lower concentrations the carotenoid exerts antioxidant activity (0.25–1 μ*M*) (20). In addition, recent observations suggested that the carotenoid may exhibit pro-oxidant properties also in the presence of different modulators of intracellular redox status such as cigarette smoke; in fact cigarette smoke-derived free radicals cleave β-carotene into many derivatives such as carbonyl-containing polyene chain cleavage products, β-carotene epoxides, and 4-nitro-β-carotene, some of which are unstable under condition of oxidative stress and can trigger a further oxidation (2, 12, 21). Our current results confirm this theory. In fact, when β-carotene 1 μ*M* was given alone to RAT-1 fibroblasts, it did not affect HO-1, whereas when administered together with TAR 25 μg/ml HO-1 protein was induced as early as 4 h of treatment. The earlier induction of HO-1 following TAR + β-carotene exposure with respect to TAR alone, provides indirect evidence on the higher degree of oxidative stress elicited by the two substances given in combination (21). Surprisingly, this initial induction was followed by a progressive decrease of HO-1 levels and, after 24 h of treatment, this protein was significantly reduced. Why was a well-known antioxidant enzyme such as HO-1 strongly repressed in the presence of a prolonged pro-oxidant attack?

This apparent paradox could be explained by the evidence that HO-1 induction is not always a useful event for cells (27). During last years, several studies demonstrated that many stressful conditions such as thermal stress, hypoxia, and interferon-γ treatment were able to repress HO-1 in many human cell lines, whereas the same stimuli induced HO-1 in rodent cells (27). The current hypothesis suggests that HO-1 repression may be useful for cells because it (a) decreases the energetic costs necessary for heme degradation, (b) prevents local accumulation of HO products, and (c) transiently increases intracellular heme pool that, in turn, regulates the expression of different genes and stimulates cell growth and differentiation (27). Keeping in mind these observations and the results shown in this paper, we hypothesized that the HO-1 overexpression after short exposure (4–6 h) of cells to TAR alone or in combination with β-carotene, can be considered as an early attempt of the cell to react to an "acute" oxidant stress. On the other hand, if the oxidant stimulus persists for a longer time (24 h), the cell represses HO-1 expression, through the activation of Bach1. This hypothesis was enforced by the evidence that the well-known antioxidant α-tocopherol efficiently counteracted the activation of Bach1 by TAR alone and, in a lesser extent, by TAR + β-carotene, thus providing a further demonstration that TAR plus the carotenoid is able to trigger an oxidative stress higher than that induced by TAR alone.

Furthermore, as mentioned above, the repression of HO-1 by Bach1 may have the purpose to avoid the toxic accumulation of iron, BR, and CO in cells. In fact we found that when RAT-1 fibroblasts undergo oxidative stress, including TAR treatment, they have a sustained increase in HO activity resulting in a marked augment in BR and CO levels in the range 0.5–1 μ*M* (data not shown). Both the bile pigment and the gas at these concentrations had a significant antiproliferative effect on rat fibroblasts in our experimental system, in good agreement with recent data in literature that show as BR and CO reduce the growth of both human fibroblasts and rat smooth muscle cells by arresting the cell cycle at the G0/G1 or G1/S transition phases (8, 23, 28, 40). In the light of these findings it is reasonable to hypothesize that when RAT-1 cells are treated with TAR + β-carotene for 24 h, which results in a significant reduction in HO-1 protein with respect to TAR alone, the amounts of BR and CO produced are lower, thus reducing their inhibitory effects on cell growth. This assumption is also strengthened by our result showing that fibroblasts exposed to TAR + β-carotene for 24 h recovered from the G0/G1 phase and were stimulated to re-enter in the S-phase and replicate.

The consequence of HO-1 repression on cell growth has been studied also in human Mv1Lu cells by using a different approach. In this human cell line we silenced the gene encoding for Bach1 using the siRNA technique, with the purpose to avoid the repression of HO-1. The results of these experiments confirmed the role of Bach1 in modulating cell growth. In fact, Mv1Lu cells, in which Bach1 gene was knocked-down, exhibited a significant decrease in cell growth only after the administration of TAR and β-carotene in association, thus reinforcing the results obtained with RAT-1 fibroblasts and demonstrating that when HO-1 is repressed, and its products reduced, the cells can grow in an uncontrolled manner. Unfortunately, Bach1 siRNA transfection did not affect Mv1Lu cell growth upon treatment with TAR alone. At this time we cannot dissect the molecular mechanism(s) that underlie these different effects on cell growth, in particular because the potential homeostatic changes occurring in cells following Bach1 knock down are still poorly understood.

Finally our data provided evidence that the HO-1 repression occurred both in rat fibroblasts and human lung carcinoma cells, thus demonstrating as HO-1 repression is an interspecies conserved mechanism by which cells can proliferate in response to tissue damage.

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ABBREVIATIONS

bp, Base pair; BR, bilirubin; CO, carbon monoxide; CO-RM, CO-releasing molecule; HO, heme oxygenase; RT–PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interference RNA; Sn-PP-IX, Sn-protoporphyrin-IX; TAR, cigarette smoke condensate.

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