Expression of c-fos in Quiescent Swiss 3T3 Cells Exposed to Aqueous Cigarette Smoke Fractions

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ABSTRACT

The exposure of quiescent Swiss 3T3 cells to mainstream cigarette smoke (CS) trapped in PBS solution (smoke-bubbled PBS) resulted in the dose-dependent expression of c-fos mRNA and protein. Kinetic investigations revealed that in contrast to mitogens, which strongly but transiently induce the c-fos promoter within minutes, c-fos transcripts in cells exposed to 0.03 puffs (—1 cm³) of CS/ml of medium accumulated slowly but were still seen after 8 h; the maximum expression rates were between 2 and 6 h of exposure. This specific expression pattern appears to be the result of altered posttranscriptional as well as transcriptional regulation, since a strikingly increased stability of the c-fos message (t½ ≥ 2 h versus <20 min in serum-stimulated cells) in smoke-treated cells was observed in addition to slight transcriptional activation of the c-fos promoter. CS-dependent DNA damage can be excluded as the only source for this altered expression pattern, since inhibition of DNA strand break formation by either catalase or o-phenanthroline had no detectable effect on the CS-induced c-fos expression. The results described here, and other CS-dependent cellular and biochemical effects, are similar to those induced in vitro by okadaic acid, a specific inhibitor of cell growth-regulatory protein phosphatases 1/2A (PP-1/2A). Hence, the effects of smoke treatment on these key enzymes were compared to those of okadaic acid based on the ability to change post-transcriptional regulation, since a specific inhibitor of cell growth-regulatory protein phosphatases 1/2A (PP-1/2A). Results from these experiments indicate that both treatments inhibited PP-1/2A in a concentration- and time-dependent manner. The data presented suggest that PP-1/2A may, at least in vitro, be targeted by water-soluble active compounds present in cigarette smoke.

INTRODUCTION

The c-fos gene belongs to the family of growth- and differentiation-related immediate early genes, the expression of which generally represents the first measurable cellular response to a variety of chemical and physical stimuli as diverse as growth factor treatment (for review see Refs. 1, 2), light (3), oxidative stress (4—6), or DNA-damaging agents (7). Hence, the effects of smoke treatment on these key enzymes were compared to those of okadaic acid based on the ability to change post-transcriptional regulation, since a specific inhibitor of cell growth-regulatory protein phosphatases 1/2A (PP-1/2A). Results from these experiments indicate that both treatments inhibited PP-1/2A in a concentration- and time-dependent manner. The data presented suggest that PP-1/2A may, at least in vitro, be targeted by water-soluble active compounds present in cigarette smoke.

MATERIALS AND METHODS

All chemicals, radiochemicals, and enzymes were obtained at the highest purity available from Sigma Chemical Co. (Deisenhofen, Germany), Amer sham (Braunschweig, Germany), and Boehringer Mannheim (Mannheim, Germany), respectively. Smoke-bubbled PBS was generated by bubbling 30 puffs of mainstream smoke (1 puff = 35 cm³ of mainstream smoke) from the University of Kentucky standard reference cigarette 2R1 through 18 ml of PBS-Dulbecco using a standard smoking procedure (28). To obtain the required concentrations, smoke-bubbled PBS (maximum, 0.36 ml) was added to 20 ml of DMEM (GIBCO-BRL, Eggenstein, Germany) without serum, and the cells were exposed immediately thereafter.

Cell Culture and Treatment. Swiss albino 3T3 mouse fibroblast cells (American Type Culture Collection CCL 92) were cultured to near confluence in 15-cm tissue culture dishes (Greiner, Frickenhausen, Germany) with 20 ml of DMEM supplemented with 4% sodium hydrogen carbonate, 1-glutamine, streptomycin, penicillin, and 10% FCS (GIBCO-BRL). Cells arrested in G0 by serum deprivation (0.5% FCS; 48 h) were generally used in these experiments. Prior to treatment, the cells were washed with serum-free DMEM and immediately thereafter exposed for the indicated incubation times to smoke-bubbled PBS containing DMEM without FCS. The viability of cells exposed to 0.03 puffs/ml was in general between 85 and 90% during the first 4 h of treatment but declined slightly to ~75% after 6 h of exposure, when concentrations higher than 0.03 puffs/ml were used.

In order to evaluate the effects of catalase and o-phenanthroline on CS-dependent c-fos expression, both compounds were added to the exposure medium prior to smoke-bubbled PBS (preincubation of o-phenanthroline; 15 min). Following exposure, cells were washed again with serum-free medium and subsequently prepared for RNA harvest.

c-fos Expression Analysis. Total RNA was isolated by standard methods (29) with the use of a commercially available RNA isolation kit (Wak-Chemie, Bad Homburg, Germany). Fifteen µg of RNA/sample were analyzed by routine methods including denaturing RNA gel electrophoresis, Northern blotting, Northern hybridization, and autoradiography (29). A 32P-labeled (30) 1-kilobase PstI/PvuII fragment of Finkel Biskis Jinkins osteosarcoma virus (Dianova, Hamburg, Germany) was used as hybridization probe.

Molecular toxicological investigations have demonstrated that water-soluble fractions of CS can cause oxidative stress in smoke-exposed cells in vitro (21). For example, CS trapped in PBS solution (smoke-bubbled PBS) induces lipid peroxidation (22), elevated expression rates of heme oxygenase (23), DNA single strand breaks due to the generation of Fenton reaction-derived hydroxyl radicals (23—27), and a decrease in the amount of protein thiols (22).

Continuing the investigations of the immediate cellular response to aqueous CS fractions, in the present study we investigated the expression of c-fos in cultured 3T3 cells following exposure to smoke-bubbled PBS. 3T3 cells served here as a model mammalian cell system which has been shown to harbor signal transduction pathways resulting in c-fos expression (2), as well as to respond to oxidative stress induced by CS (23). Altered regulation of the c-fos gene at the transcriptional as well as posttranscriptional level was observed. This finding, in concert with other CS-induced cellular effects, parallels findings described for the PP-1/2A-specific inhibitor okadaic acid (for review see Refs. 18 and 20). In fact, follow-up experiments provided further evidence that these protein phosphatases are a target of active compounds in aqueous CS fractions in vitro.
was performed by treating the cells with actinomycin D (25 mg/ml) and with the use of a commercially available RNA isolation kit (Wak-Chemie).

Evaluation of the half-life of c-fos mRNA either in serum-stimulated (20% FCS; 0.5 h) or in smoke-bubbled PBS-exposed cells (0.03 puffs CS/ml; 4 h) was performed by treating the cells with actinomycin D (25 mg/ml) and determining the amount of c-fos mRNA up to 120 min later by standard Northern blot analysis. The hybridization signals were quantified by densitometric scanning (Hirschmann, Unterhaching, Germany) or by the use of a phosphor imager (Molecular Dynamics, Krefeld, Germany).

**Determination of PP-1/2A Activity.** Cellular extracts were prepared from cells exposed for the indicated times and concentrations either to smoke-bubbled PBS or okadaic acid (GIBCO-BRL). Preparation of extracts as well as determination of PP-1/2A activity in these extracts by their ability to dephosphorylate 32P-labeled glycogen phosphorylase a (32) were performed with the use of a commercially available protein phosphatase assay system (GIBCO-BRL). Released 32P-labeled phosphate was quantitatively determined in a liquid scintillation counter (Packard, Frankfurt, Germany). For evaluation, results were standardized to the protein concentration present in the extracts. Protein determination was performed with the use of a modified Lowry method (33).

**Immunofluorescence Staining.** Immunofluorescence staining of c-Fos in nuclei from quiescent cells or quiescent cells exposed to either 0.03 and 0.045

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**RESULTS**

**Smoke-bubbled PBS Induces c-fos Expression.** Northern blot analysis of quiescent cells (0.5% FCS; 48 h) exposed for 2 h to smoke-bubbled PBS revealed a dose-dependent expression of the c-fos proto-oncogene (Fig. 1). The dose-response curve (Fig. 1B) indicates a threshold level at a dose of approximately 0.015 puffs/ml. The decrease of detectable c-fos mRNA at smoke-bubbled PBS concentrations exceeding 0.06 puffs CS/ml may result from increasing genotoxicity. On the basis of these results, experiments described below were performed at a concentration of 0.03 puffs CS/ml.

Investigations of the time dependence of c-fos mRNA expression in smoke-bubbled PBS-treated cells revealed a slow accumulation of the transcript, a significant hybridization signal first detectable after 30 min of exposure (Fig. 2A). Maximum levels of c-fos mRNA were detected between 2 and 6 h of exposure. This pattern is significantly different from that observed in response to other external stimuli such as growth factors or tumor promoters of the phorbol ester type, which strongly induce c-fos within about 15 min, followed by an immediate return to basal expression levels (Fig. 2B) (2, 15). However, the pattern of c-fos expression in CS-treated cells is similar to that observed in cells treated with okadaic acid, a specific inhibitor of Ser/Thr-PP-1/2A (20).

Immunofluorescent staining of smoke-bubbled PBS-treated cells with the use of a murine Fos-specific antibody showed a dose-dependent increase in labeling of the nuclei in a range between that observed in high serum-stimulated and untreated starved cells (Fig. 3), with the exception that the fluorochrome used was extravidine-conjugated FITC in PBS containing 1 mg/ml BSA and 0.5% NP40.

**Smoke-bubbled PBS Exposure Affects c-fos Expression at a Transcriptional as Well as a Posttranscriptional Level.** In order to analyze c-fos expression in cells treated with smoke-bubbled PBS in more detail, the transcriptional activity of the c-fos promoter, as well

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Fig. 1. Expression of the c-fos proto-oncogene in smoke-bubbled (sb) PBS-treated quiescent cells. Cells (4 × 10⁵) were exposed for 2 h in serum-free DMEM containing smoke-bubbled PBS concentrations as indicated. A, Northern blot; B, quantitative evaluation. Preparation of total RNA, electrophoresis, and Northern blotting were performed as described in "Materials and Methods." Quantitation was achieved by phosphor-imaging of hybridization signals. kb, kilobase.

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Fig. 2. Kinetics of c-fos expression in quiescent 3T3 fibroblasts exposed to 0.03 puffs/ml smoke-bubbled PBS (A) or stimulated with 20% PCS (B). Cells were exposed to smoke-bubbled PBS for the indicated times and analyzed for c-fos expression as described in Fig. 1. kb, kilobase.
Fig. 3. C-Fos expression in control cells (A), cells exposed to 0.03 (B) or 0.045 (C) puffs/ml smoke-bubbled PBS for 5 h, or cells stimulated with 20% FCS for 1 h (D). Immunofluorescence staining was performed according to Ref. 20.

as the half-life of c-fos transcripts, was determined in quiescent 3T3 cells exposed to 0.03 puffs CS/ml. The results were compared to those described in the literature for high serum-stimulated cells.

Nuclear run-on analysis of smoke-bubbled PBS-treated cells revealed that the transcriptional activity of the c-fos promoter slightly increased after approximately 30 min of exposure and began returning to background levels thereafter (Fig. 4). This pattern of c-fos expression contrasts with the well documented rapid and strong activation of the c-fos promoter in serum- or growth factor-stimulated cells (12-14). In addition, Fig. 4 shows the previously described gradual but strong increase in heme oxygenase transcription in smoke-bubbled PBS-treated cells (23) as control.

The stability of c-fos transcripts in cells exposed for 4 h to smoke-bubbled PBS was evaluated by blocking transcription with the use of actinomycin D and determining the amount of c-fos mRNA up to 2 h thereafter (Fig. 5). The half-life of c-fos transcripts was evaluated by semi-logarithmically plotting the amount of c-fos mRNA versus the exposure time to actinomycin D. As seen in Fig. 5C, smoke-bubbled PBS increases the half-life of c-fos mRNA to ~2 h. In comparison, the half-life of c-fos mRNA in cells stimulated by high concentrations of serum is <20 min, which is in accordance with published data (2, 15). In this context, it should be noted that another species of mRNA, i.e., heme oxygenase mRNA, is processed normally in smoke-bubbled PBS-treated cells (23). Thus, these results clearly demonstrate that water-soluble compounds in CS interfere with the regulation of c-fos expression on a transcriptional as well as a posttranscriptional level by slightly inducing the c-fos promoter and strongly stabilizing c-fos transcripts.

DNA Damage Is Not the Major Signal for Expression and Persistence of c-fos mRNA in Smoke-bubbled PBS-Treated Cells. There is now conclusive evidence that most of the DNA damage observed in smoke-bubbled PBS-treated cells is attributable to the
formation of hydroxyl radicals via the Fenton reaction (23–27). In order to determine whether DNA damage is the reason for the appearance and persistence of c-fos mRNA in smoke-bubbled PBS-treated cells as reported for other DNA-damaging agents (7), exposure was performed in the presence of either catalase or the membrane-permeable iron-chelating agent o-phenanthroline (34) (Fig. 6). Although both inhibitors of Fenton-type hydroxyl radical formation were used at concentrations known to significantly inhibit DNA damage resulting from exposure to smoke-bubbled PBS (23), neither compound was able to suppress or even reduce CS-dependent c-fos expression (Fig. 6). Even preincubation of smoke-bubbled PBS with catalase showed no inhibitory effect (Fig. 6A). Thus, these data indicate that DNA damage is not the main signal for c-fos expression in smoke-bubbled PBS-treated cells.

**Smoke-bubbled PBS Partially Inhibits PP-1/2A.** Since the cellular effects of water-soluble CS fractions described here and in a previous study (23) strikingly parallel those of the PP-1/2A-specific inhibitor okadaic acid (20), the enzymatic activity of PP-1/2A was determined in cellular extracts prepared from cells exposed to smoke-bubbled PBS or treated with okadaic acid.

As shown in Fig. 7, smoke-bubbled PBS treatment leads to a time- and concentration-dependent decrease in PP-1/2A enzymatic activity. In comparison to the inhibition observed in the presence of 0.5 μM okadaic acid, exposure to smoke-bubbled PBS at 0.03 puffs CS/ml was slightly less effective, and at 0.1 puffs CS/ml the effect was similar. In contrast, no effect of high serum treatment on PP1/2A enzyme activity in 3T3 cells could be detected within the interval of 4 h (data not shown). Moreover, the time-dependent inhibition of PP-1/2A by smoke-bubbled PBS and okadaic acid indicates that both test materials require nearly 4 h to fully express their effects (Fig. 7A). Hence, according to these results, PP-1/2A may represent targets for active compounds present in smoke-bubbled PBS.

**DISCUSSION**

In the present study, the expression of the c-fos proto-oncogene in quiescent Swiss 3T3 cells following exposure to aqueous CS fractions is reported. This gene is also expressed upon CS exposure of naturally growing cells, although 2–3 times higher concentrations of aqueous CS fractions were required in these cells (data not shown). The c-fos gene was chosen as a marker for an early response of a cell to external stimuli. Previous studies have demonstrated that exposure of Swiss 3T3 cells to smoke-bubbled PBS leads to a transient decrease in the translation efficiency (23). Since inhibition of protein synthesis triggers a strong increase in c-fos transcripts, based on transcriptional activation of the c-fos promoter as well as on stabilization of the otherwise very short-lived c-fos message (19, 35, 36), it was tempting...
to conclude that c-fos expression in smoke-bubbled PBS-treated cells might result from partial inhibition of protein synthesis. However, this effect can be excluded as the only explanation for c-fos expression in smoke-bubbled PBS-treated cells for the following reason: c-fos expression and mRNA persistence induced by protein synthesis inhibitors are completely reversible within 10 min after removal of the inhibitor from the medium (35). In smoke-bubbled PBS-treated cells, the CS-dependent decrease in translation efficiency completely returns to and even exceeds basal protein synthesis rates after 4 h of exposure (23). This observation is confirmed by the detection of increased amounts of Fos protein in nuclei of cells exposed for 5 h to smoke-bubbled PBS (Fig. 3). Thus, if elevated amounts of c-fos mRNA in CS-treated cells were caused mainly by inhibition of protein synthesis, it could be expected that c-fos transcripts would then again be the target of rapid “turnover” regulation. However, as shown in Figs. 2 and 3, c-fos mRNA is still present and subject to protein synthesis in cells exposed for 5 h, exhibiting half-lives of >2 h (Fig. 5). In this context, it is worth mentioning, however, that a true inhibitor of protein synthesis (i.e., anisomycin) at inhibitory but also at subinhibitory concentrations triggers prolonged immediate early gene expression (37) similar to the effects reported for CS in this study. Most interestingly, the anisomycin-induced pathway can also be activated by UV irradiation as well as by okadaic acid. Further studies will show whether the same signal transduction pathway is used by anisomycin as well as by CS.

Recent studies revealed that hydroxyl radicals formed via the iron-catalyzed Fenton reaction (38, 39) are mainly responsible for the genotoxic activity observed in cells exposed to aqueous extracts of CS (23–27). Moreover, hydroxyl radicals have been shown to induce immediate early genes like c-fos and c-myc with kinetics very similar to the rapid and transient expression pattern observed in response to growth factor stimulation (4, 5). However, the kinetics of c-fos expression in smoke-bubbled PBS-treated cells were characterized by a slow accumulation and persistence of c-fos transcripts (Fig. 2), which were shown to result from slight activation of the c-fos promoter combined with strong stabilization of the c-fos message (Figs. 4 and 5).

Induction of c-fos and persistence of c-fos mRNA up to 16 h after treatment have recently been reported for a variety of DNA-damaging agents (7). Since it is also known that smoke-bubbled PBS induces DNA strand breaks in vitro (23–27), this may suggest that DNA damage is also a signal for c-fos expression in smoke-bubbled PBS-treated cells. However, inhibition of DNA strand breaks either by catalase or by o-phenanthroline had no influence on the CS-dependent c-fos expression (Fig. 6). Moreover, this result corroborates the conclusion that hydroxyl radicals produced by the Fenton reaction are not involved in CS-dependent alteration in the expression of this gene.

Since CS is a complex chemical mixture, it is conceivable that the specific pattern of c-fos expression in CS-treated cells may be caused by independent effects, e.g., one leading to an increased c-fos transcription rate, while another may target the c-fos mRNA-degrading machinery. However, another possibility which should be considered refers to the observation that the cellular effects induced by smoke-bubbled PBS parallel those induced by okadaic acid, a specific inhibitor of Ser/Thr-PP-1/2A (for review see Ref. 18). In particular, the following similarities were detected in cultured fibroblasts following exposure to either compound: (a) the pattern of c-fos expression, i.e., the activation of the c-fos promoter as well as the stabilization of c-fos mRNA (Ref. 20; Figs. 4 and 5); (b) a transient decrease in protein synthesis (20, 23); and (c) an extensive stress response as expressed, e.g., by elevated heme oxygenase expression levels for smoke-bubbled PBS (23) as well as okadaic acid. Moreover, (d) smoke-bubbled PBS, as well as okadaic acid (40), induces cell cycle arrest in target cells in vitro; and finally and most evidently, (e) both materials lead to the inhibition of PP-1/2A enzymatic activities (Fig. 7).

A possible candidate present in CS that may specifically lead to the inhibition of protein phosphatases in smoke-bubbled PBS-treated cells is NO. Hemmings et al. (41) and Tsou et al. (42) demonstrated that this type of gaseous messenger inhibits protein phosphatase 1 via a NO/cyclic GMP-induced pathway in neuronal cells. On the other hand, smoke-bubbled PBS may induce its inhibitory effects on PP-1/2A by a more unspecific mechanism. For example, it has recently been shown that water-soluble components of CS target free sulfhydryl groups, because a rapid decrease in free glutathione levels was observed in smoke-bubbled PBS-treated cells that did not occur when cysteine was added to the medium (23). Thus, the PP-1/2A-inhibitory effect of smoke-bubbled PBS may also be derived from the inactivation of essential sulfhydryl groups present in these enzymes.

Whether NO or the sulfhydryl reactivity alone or in combination with other yet unidentified water-soluble components and reaction products of CS are responsible for the effects described here remains to be elucidated. The physiological consequences of c-fos expression...
and the partial inhibition of PP-1/2A in smoke-bubbled PBS-treated cells will also be the subject of further investigations. Finally, the relevance of these in vitro data for the in vivo situation has to be evaluated.

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