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Nuclear accumulation and up-regulation of p53 and its associated proteins after H_2S treatment in human lung fibroblasts

Dear Editor:

In recent years, it has become clear that hydrogen sulphide (H_2S) plays a number of biological roles and may function as a novel gasotransmitter in the body alongside nitric oxide (NO) and carbon monoxide (CO) [1]. H_2S is synthesized naturally from L-cysteine in mammalian tissues in a reaction catalysed by two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS) [2]. Recently, attention has been focused on the potential physiological and pathophysiological role of H_2S in the body. It has been shown, for example, H_2S plays a role in the regulation of vascular function both in health and disease [3–5]. Interestingly, H_2S is also produced naturally at sites of inflammation and it is known that chronic inflammation is associated with uncontrolled cellular proliferation. However, in contrast to other gasotransmitter (*e.g.* NO) very little information exists on the mechanism by which H_2S influences cell growth. In the present study, we have attempted to assess the biological effects of H_2S in normal human lung fibroblast (MRC-5) cells.

Recently, we have reported that H_2S treatment increased cell death, formation of micronuclei (MN) and alteration in cell cycle [6]. Broadly, similar conclusions were also reported using single cell gel electrophoresis (SCGE) to show that sodium sulphide (Na_2S: 250 μM/L) caused radical-associated DNA damage in the Chinese hamster ovary (CHO) cells [7]. Together, these data indicate that H_2S is a potent clastogenic agent and suggest that it has a role in DNA damage-induced responses. In the present study, we have intended to understand the mechanism(s) involved in the genomic instability caused by H_2S. The tumour suppressor protein p53 plays a key role in maintaining genomic integrity by controlling cell-cycle progression and cell survival [8]. Cells under stresses such as DNA damage, hypoxia and aberrant oncogene signals trigger the tumour suppressor protein p53, which transcribes genes that induce cell-cycle arrest, DNA repair and apoptosis [8, 9]. The mechanisms by which H_2S up-regulates p53 and thereby induces DNA damage and alters cell-cycle progression remain unclear. In the present study, we report that the up-regulation of both the inducer protein p53 and the effector protein p21 in normal lung fibroblast cells several hours after 50 μM of NaHS (donor of H_2S) treatment followed by the key proteins involved in cell cycle, *i.e.* Cyclin A, Cyclin E (a trend for CDC-6, p16 to increase) and decrease in Cyclin D, with a trend for p27 to decline (Fig. 1A). Interestingly, down-regulation of p27 and Cyclin D coincided with that of the H_2S-induced growth arrest reported in our previous study, suggesting that these proteins may have a role in mediating the H_2S induced cell cycle arrest. Furthermore, in the $p53^{-/-}$ MEF cells showed up-regulation of Cyclin D following the NaHS treatment (Fig. 1B) suggests that p53 is essential to maintain the genomic integrity of the NaHS treated cells. In addition, immunofluorescence studies were performed to better understanding the activation of p53 by H_2S. Cells were stained with a specific antibody of p53. The results showed a higher expression of p53 in the nucleus of the H_2S treated cells (Fig. 1F).

Further, we determined whether the activation of p53 and p21 was attributed to the increase in protein stability. Cells were either treated or untreated with NaHS. Six hours after NaHS treatment, cells were treated with cycloheximide (CHX, 10 μg/ml) to inhibit the *de novo* protein synthesis. p53 and p21 steady state levels were monitored at various time-points after CHX addition (Fig. 1C). The rate at which p53 and p21 levels decreased under these conditions was measured as protein stability. In the untreated cells, p53 and p21 levels were decreased following CHX treatment. In contrast to H_2S-treated cells, p53 level is elevated for up to 6 hrs following CHX treatment. However, CHX treatment blocks the p21 expression. In the present study, we investigated the subcellular expression of p53 followed by the H_2S treatment. Cells treated earlier with NaHS and followed by CHX treatment were treated earlier with NaHS for 6 hrs were sampled at the indicated time-points and subjected to cytoplasmic (Fig. 1D) and nuclear (Fig. 1E) fractions as explained earlier [10, 11]. In the unstressed cells, cytoplasm is the exclusive site of p53 degradation, and therefore nuclear export of p53 is prerequisite for its delivery to cytoplasmic proteasomes. In the present study, steady state decrease in p53 level was only evident in nuclear fraction, indicating that NaHS treatment accumulates p53 in the nucleus. In conclusion, we demonstrate for the first time that the H_2S-induced increase in p53 level is due to an increase in p53 protein stability. These results are of significance as they uncover a hitherto unknown and a potentially fundamental role for H_2S in determining the cell fate and may provide for development of a novel therapeutic approach to the regulation of cell growth.

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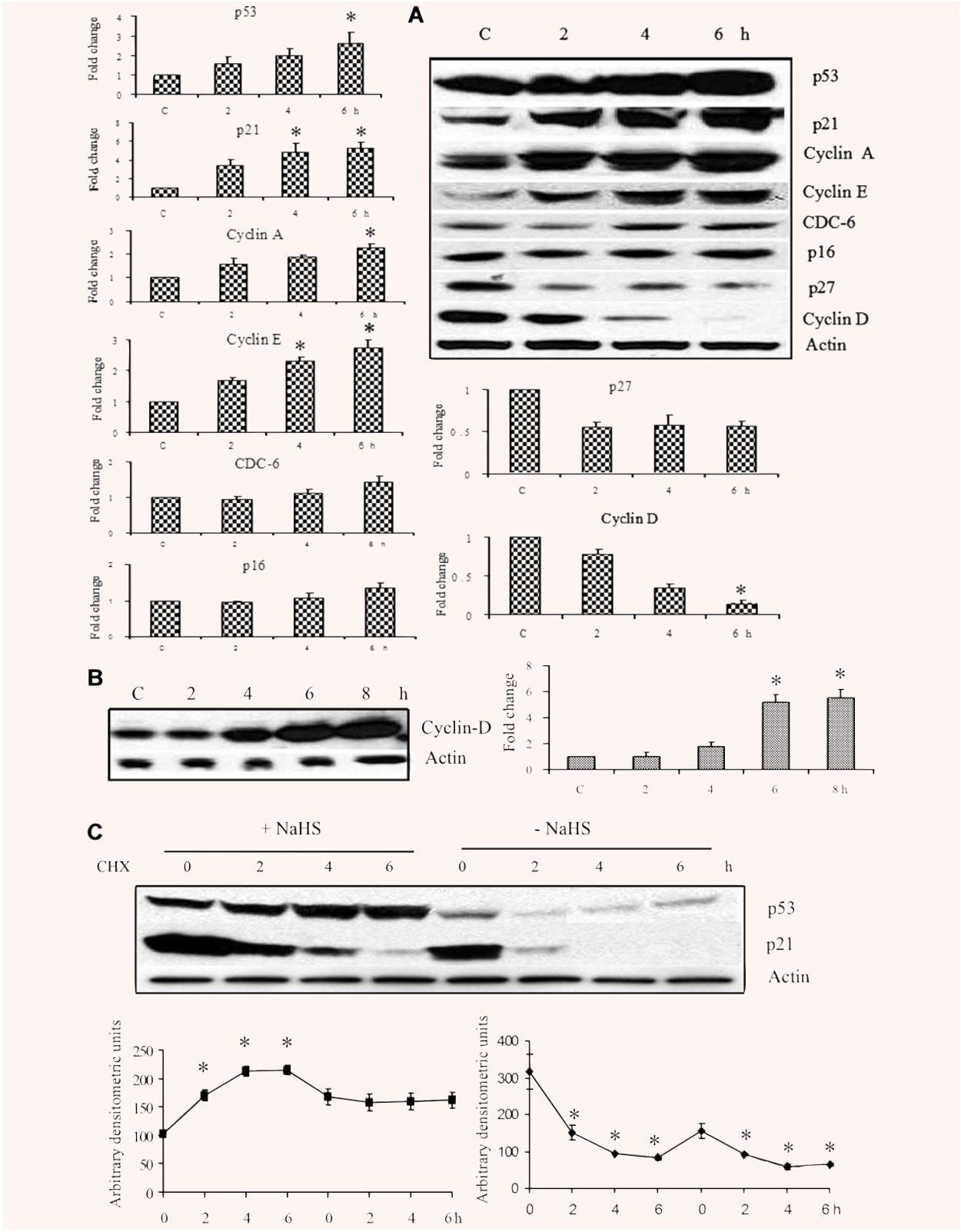

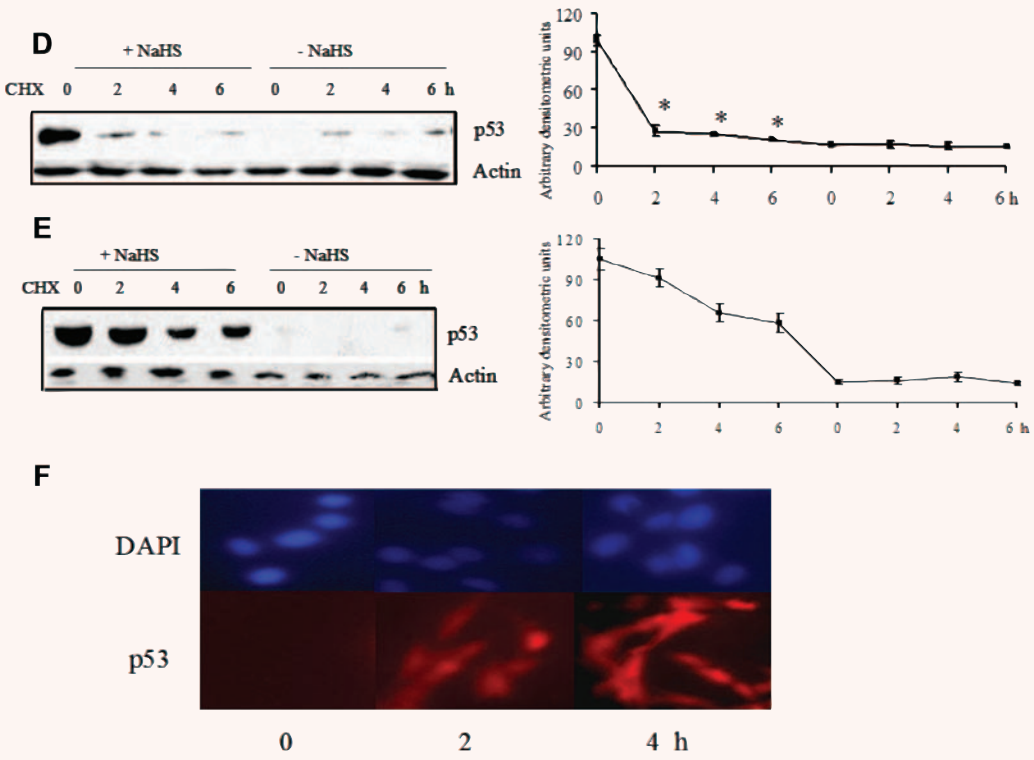

Fig. 1 H_2S-induced p53 is transcriptionally active. In the Western blot studies, whole cell or subcellular extracts were prepared at the indicated time-points and thereafter equal amount of proteins (25–50 μg) were separated by 4–20% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane and immunoreacted with antibodies. The densities of the bands were quantified using Kodak 1D image analysis software. Bar graphs show fold induction/change. All values are expressed as mean ± S.E.M. Each value is the mean of at least three separate experiments. Significant differences between test and control data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. A value of $P < 0.05$ was considered statistically significant compared with the representative control. (**A**) In the present study, we report the up-regulation of both the inducer protein p53 and the effector protein p21 in normal lung fibroblasts several hours after 50 μM of NaHS (donor of H_2S) treatment followed by the key proteins involved in cell cycle, *i.e.* Cyclin A, Cyclin E (a trend for CDC-6, p16 to increase) and decrease in Cyclin D, with a trend for p27 to decline. (**B**) $p53^{-/-}$ MEF cells showed up-regulation of Cyclin D. (**C**) H_2S induces p53 protein stabilization. Cells were incubated for 6 hrs either in the presence or absence of H_2S. At the end of 6-hr incubation, cycloheximide (CHX) was added to all cultures at a final concentration of 10 μg/ml. Cells were then sampled at 0, 2, 4 and 6 hrs and steady state levels of p53 and p21 in whole cell lysates were assessed by Western blot analysis. Nuclear degradation of p53 occurs after NaHS treatment. (**D**) cytoplasmic and (**E**) nuclear fractionation of MRC-5 cells. p53 degradation was assessed by Western blot analysis. After CHX treatment in which cells were treated NaHS 6 hrs before, cells were sampled at the indicated time-points and subjected to cytoplasmic and nuclear fractions. Actin was used as loading control. (**F**) Cellular p53 was examined by immunofluorescence staining with the p53 antibody (DO-1). Nucleus was visualized by staining with 4,6-diamino-2-phenylindole (DAPI). Cells were harvested for p53 immunofluorescence staining at the times indicated.

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