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Effects of Benzaldehyde on Protein Metabolism of Human Cells Cultivated *in Vitro**

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Abstract—*The mechanism by which the antitumour agent benzaldehyde inhibits cell growth has been investigated. Human NHIK 3025 cells were synchronized by selection of mitotic cells and the protein content at various stages of the cell cycle was recorded by use of flow cytometry. In the presence of benzaldehyde (concentrations above 0.5 mM, ~50 μg/ml) the rate of protein accumulation was reduced to the same extent throughout the cell cycle. The rates of protein synthesis and protein degradation were measured by incorporation and release, respectively, of radioactively labelled valine in exponentially growing cells. It was found that benzaldehyde primarily reduced the rate of protein synthesis, while it induced only a very small effect (reduction) on the rate of protein degradation. When comparing the rate of cell-cycle progression with the rate of protein accumulation, it was found that the median interphase duration was equal to the protein doubling time even for concentrations of benzaldehyde giving a marked reduction in the rate of protein accumulation. Similar results have been observed on these cells using the specific protein synthesis inhibitor cycloheximide. However, the two drugs have different effects during mitosis, since benzaldehyde but not cycloheximide induces a specific mitotic inhibition. It is, therefore, possible that benzaldehyde inhibits the protein synthesis by a mechanism different from that of cycloheximide, a mechanism which simultaneously results in a specific mitotic inhibition. A hypothesis is proposed on the mechanism of action of benzaldehyde: that the drug might inhibit a process in the cells which activates enzymes. Such an effect might possibly entail a reduced protein synthesis as well as a prolonged mitosis. In addition, it might also count for the reported de-transforming activity of benzaldehyde on malignant cells.*

INTRODUCTION

BENZALDEHYDE has been reported to induce antitumour effects on various tumours in both mice [1] and humans [2].

Histological observations of human tumours after long-term treatment with benzaldehyde was reported to indicate a change towards higher differentiation [2]. However, little is known about the effects of benzaldehyde at the cellular level. In a previous report [3] we presented data on inactivating and cell-cycle inhibitory effects of benzaldehyde on synchronized cells of the established human cell line NHIK 3025 cultivated *in vitro*. The results showed that benzaldehyde reduced the rate of cell-cycle progression (cell-cycle inhibitory effect) to about the same degree in G_1, S and G_2. With respect to both inhibitory and inactivating effects, the cells were particularly sensitive in mitosis.

The effect of benzaldehyde on cell-cycle progression was found to be very similar to that induced on the same cells by the specific protein synthesis inhibitor cycloheximide [3, 4]. It has furthermore been shown that certain aliphatic aldehydes can depress protein synthesis [5–7]. We therefore found it interesting to study the effect of benzaldehyde on the protein metabolism of NHIK 3025 cells and to compare the effects of benzaldehyde on protein metabolism and on cell-cycle kinetics in order to see whether or not the cell-cycle inhibition induced by benzaldehyde might be a secondary effect of a reduced accumulation of protein.

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MATERIALS AND METHODS

Cells and synchronization

Human cells, NHIK 3025, established from a cervical carcinoma *in situ* [8, 9], were cultivated as monolayers in medium E2a [10] supplemented with 20% human serum (prepared in the laboratory) and 10% horse serum (Gibco Europe, U.K.). The cells were routinely recultured 3 times a week. No significant delay in growth is seen after reculturing of the cells. The median cell-cycle time is 18 hr, as long as the cells are not allowed to grow to confluence [11]. The cells meet the requirements set up by Anderson *et al.* [12] for cells in balanced growth.

When synchronized by mitotic selection, the NHIK 3025 cells have the same median cell cycle time as in an exponentially growing culture. Immediately after selection the mitotic index was above 94% [11], and the synchronization index as defined by Engelberg [13, 14] was 95% for the first and 64% for the second division after selection [11]. Under growth conditions as used here the cells have median G_1, S and G_2 durations of ~6.5, ~8 and ~2.5 hr respectively, while mitosis lasts for ~1 hr [11].

Measurement of cell-cycle kinetics of synchronized cells

After mitotic selection the cells were seeded in Falcon plastic flasks (75 or 25 cm^2) with about 2×10^4 mitoses per 75 cm^2 flask and 5×10^3 mitoses per 25 cm^2 flask. After about 1 hr all the cells had completed division and attached to the bottom of the flasks.

The cells in the 75-cm^2 flasks were used to determine the time of entrance into S. The cells were trypsinized at various times after mitotic selection to be fixed and stained [15] with mithramycin (Pfizer, U.S.A.) for flow-cytometric measurements of the amount of DNA per cell. A laboratory-built flow cytometer was used [3]. From the DNA histograms the fraction of cells in G_1 was determined as described earlier [16]. In each experiment the time course of the fraction of cells in G_1 was determined both for untreated cells and for cells treated with benzaldehyde. Since benzaldehyde is added 2 hr after selection when the cells are in early G_1, a delay of entry into S represents a prolongation of only G_1.

The cells in the 25-cm^2 flasks were used to determine the time of division of individual cells as described in our previous report [3]. Briefly, fields were delineated on the bottom of the flasks and all cells within the field were marked on a map representing the field. By direct observation in an inverted microscope, the time of division was recorded for each individual cell marked on the map. About 100 cells in each flask were observed to determine the distribution of cell-cycle times. The median duration of the cell cycle or of the particular phases are defined and determined as described in Appendix II of [11].

Measurement of amount of protein in individual cells

The relative amount of protein per cell was found by analysis of protein histograms recorded on the flow cytometer. In this case the cells were fixed at 0°C with glutaraldehyde (TAAB Laboratories, U.K.) in aqueous solution (2%) for 15 min and stained with 0.4 μg/ml fluoresceinisothiocyanate (FITC, BBL, Beckton Dickinson, U.S.A.) in phosphate-buffered saline (pH 7.2). For comparison between the relative amount of protein recorded in different histograms fluorescent latex particles (Polyscience, U.S.A.) were mixed with the cell suspension before recording and the amplification of the instrument was adjusted such that the particle peak was recorded in the same channel in each histogram. The mean relative protein content of the cells was taken as the peak channel number of the cells relative to the peak channel number of the particles.

Measurement of protein metabolism in cell populations

The rates of protein synthesis and protein degradation were calculated as described previously [17]. Briefly, cellular protein was labelled to saturation during a 2-day pre-incubation with L-[U-^{14}C]-valine (Amersham, U.K.) of constant specific radioactivity (0.2 or 0.5 Ci/mol) prior to the experiment. This was achieved by using a high concentration of valine (1.0 mM) in the medium [18]. At this concentration of valine the dilution of [^{14}C]-valine by intracellular and proteolytically generated valine will be negligible [18], thus keeping the specific radioactivity at a constant level. The rate of protein degradation was calculated from the release of acid-soluble radioactivity to the medium during 1 hr of incubation in medium containing 1.0 mM unlabelled valine, thus preventing reincorporation of isotope [18]. The rate of protein synthesis was calculated from the incorporation of L-[3,4-^{3}H]-valine (Amersham, U.K.) of constant specific radioactivity (10 Ci/mol, 1.0 mM). Both release and incorporation measurements were related to the total ^{14}C-radioactivity in protein at the beginning of the respective measurement periods and expressed as %/hr [17].

Benzaldehyde

The chemical used in these experiments was purchased from Koch-Light Laboratories, U.K.

RESULTS

To test whether benzaldehyde can reduce the rate of protein accumulation of NHIK 3025 cells the relative amount of protein was measured in synchronized cells at various times after mitotic selection by use of a flow cytometer (see Materials and Methods). Figure 1 shows protein histograms of 4 different populations of synchronized cells. The two upper histograms represent untreated cells, while the two lower histograms represent populations grown in the presence of 3.2 mM benzaldehyde, which was added 2 hr after selection. In these histograms the channel number is proportional to the amount of protein per cell. The two vertical lines drawn at channel Nos 40 and 80 are reference lines, representing the mean protein content at the start and the end of the cell cycle respectively. Due to the symmetry of the peaks the maximum of each histogram is a close approximation for the mean channel No. and is thus used as a measure of the mean protein content of the population. At 10 hr the mean relative protein content of the control is 60, while the corresponding value for the benzaldehyde-treated population is 48. After 17 hr some of the control cells have divided, as demonstrated by the shoulder on the left-hand side of the histogram (upper right), representing cells in early G_1, while the mean relative protein content of the benzaldehyde-treated population is 58. Thus by 17 hr after selection the population grown in presence of benzaldehyde has about the same protein content per cell as the controls have 10 hr after selection, showing that the rate of protein accumulation is greatly reduced in presence of 3.2 mM benzaldehyde.

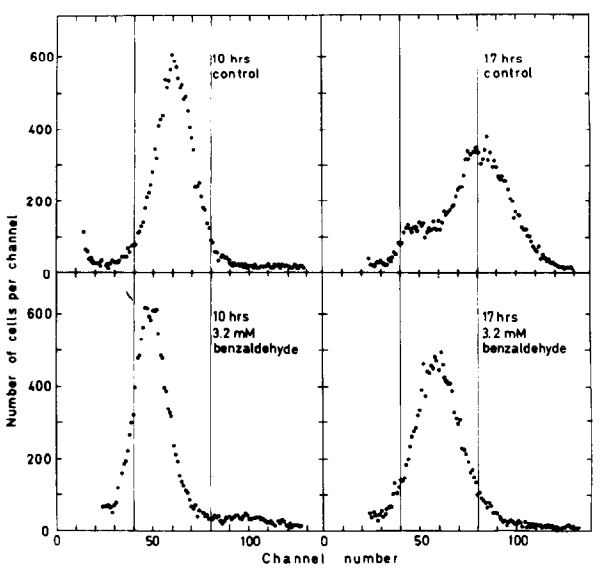

Fig. 1. Protein histograms of synchronized NHIK 3025 cells fixed 10 or 17 hr after mitotic selection for untreated cells (upper panels) and for cells grown in presence of 3.2 mM benzaldehyde from 2 hr after selection (lower panels). The channel number (abscissa) is proportional to the fluorescent light and therefore also proportional to the amount of protein per cell. The vertical bars at channel Nos 40 and 80 represent the mean protein content per cell at the start and the end of the cell cycle respectively. The rightwards displacement of the peak channel with time after mitotic selection indicates the increase in mean protein content per cell.

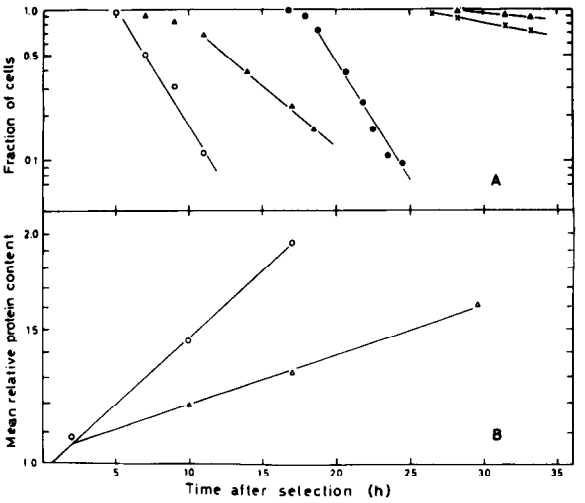

Fig. 2. (A) The fraction of NHIK 3025 cells in G_1 (○,△) and the fraction of cells that have not divided (●,▲) as a function of time after mitotic selection. Circles represent untreated control and triangles represent cells grown in presence of 3.2 mM benzaldehyde, which was added 2 hr after mitotic selection (i.e. in early G_1). Also shown is the fraction of cells that have not entered mitosis (×) as a function of the time after mitotic selection for the cell population. (B) The mean amount of protein per cell as a function of the time after mitotic selection for untreated cells (○) and cells grown in the presence of 3.2 mM benzaldehyde, which was added 2 hr after mitotic selection (△). In both (A) and (B) the ordinate axis is logarithmic. The straight lines were fitted by the method of least squares.

In Fig. 2 the increase in protein content through the cell cycle (B) is compared with the cell-cycle progression both for untreated cells and for cells treated with 3.2 mM benzaldehyde from 2 hr after mitotic selection (A). The mean relative protein content is taken as the peak channel No. of histograms like those shown in Fig. 1.

The increase in mean protein content (Fig. 2B) is exponential (the ordinate is logarithmic), with a doubling time of about 17 and 43 hr for the untreated and the treated populations respectively. From Fig. 2(A) the corresponding median cell-cycle times were found to be about 19 and 46 hr respectively.

Thus, Fig. 2(A) and (B) indicate a close correlation between the protein doubling time and the cell-cycle duration for cells grown in the absence as well as for cells grown in the presence of 3.2 mM benzaldehyde. To investigate by which mechanism benzaldehyde reduced the rate of

protein accumulation, the effects of benzaldehyde on protein synthesis and protein degradation were studied separately.

Exponentially growing cells were treated with 3.2 mM benzaldehyde for 8 hr. The incorporation of [^{3}H]-valine was measured at 4 different intervals, *viz.* the first hour after addition, the last hour before removal and the first and fifth hours after removal of benzaldehyde. In Fig. 3(A) the results are shown together with similar results for untreated cells measured simultaneously. The data demonstrate that the rate of protein synthesis is reduced from 4.8 ± 0.1 to 1.7 ± 0.1%/hr as measured during the first hour after addition of the chemical. Probably the reduction in protein synthesis rate takes place almost instantaneously, or at least within a few minutes, since the level was unchanged when measured during the eighth hour after addition. After removal of benzaldehyde the rate of protein synthesis increases rapidly back to the level of untreated cells.

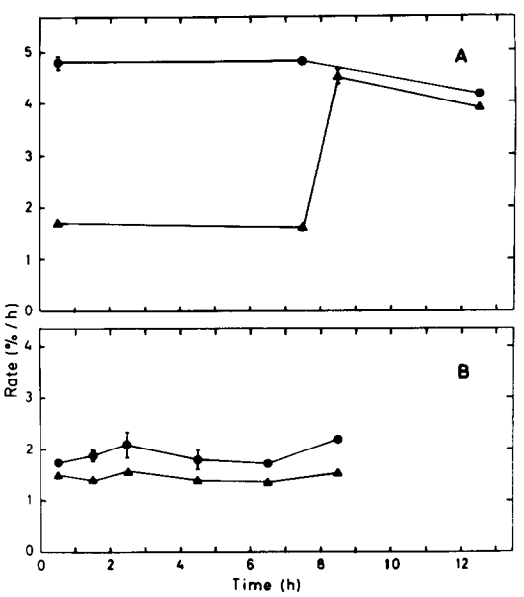

Fig. 3. (A) The rate of protein synthesis and (B) protein degradation as a function of time after addition of benzaldehyde to exponentially growing (asynchronous) NHIK 3025 cells. Data are shown both for untreated cells (●) and for cells grown in the presence of 3.2 mM benzaldehyde from 0 to 8 hr (▲). The rates are measured as the amount of protein synthesized or degraded per hour in a percentage of the total amount of protein per cell.

During the 8-hr treatment with benzaldehyde the rate of release of [^{14}C]-valine from both treated and untreated cells labelled to saturation was also measured. The data (Fig. 3B) indicate a small reduction in the rate of protein degradation in presence of 3.2 mM benzaldehyde from 1.94 ± 0.07 to 1.48 ± 0.03%/hr, i.e. about 23% reduction.

Measurements like those shown in Fig. 3(A) were also performed with cells grown in the presence of lower concentrations of benzaldehyde. The protein doubling time (T_D) was in each case calculated from the formula:

$$T_D = \frac{\ln 2}{R_s - R_d},$$

where R_s is the rate of protein synthesis and R_d is the rate of protein degradation. R_d was not measured separately, however, since it was influenced so little, even by the highest concentration of 3.2 mM. The control value for R_d was used for all concentrations of benzaldehyde below 3.2 mM and a value 23% lower was used for 3.2 mM.

The results, shown in Fig. 4, indicate that there is good correlation between the cell-cycle duration (broken line) and the protein doubling time within the range of benzaldehyde concentrations used here.

DISCUSSION

The present results demonstrate that benzaldehyde at concentrations above 0.5 mM (~50 μg/ml) is an inhibitor of the protein synthesis in NHIK 3025 cells (see Figs 1–3). The effect on protein degradation is small, even for a concentration of 3.2 mM, yet a significant reduction of the degradation rate was seen at this concentration (Fig. 3B).

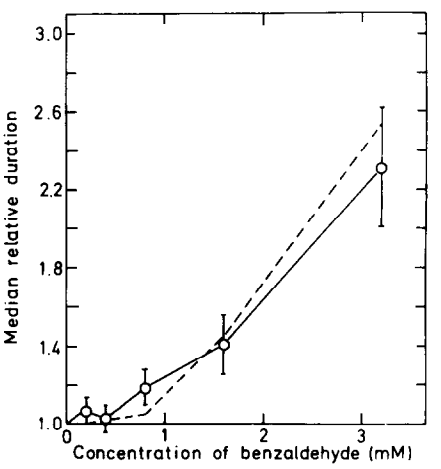

Fig. 4. The median relative doubling time for the protein content per cell (○) as a function of the concentration of benzaldehyde for synchronized NHIK 3025 cells. Benzaldehyde was added 2 hr after mitotic selection, when the cells had attached to the culture dishes and were in early G_1. Therefore the median relative doubling time in this case is calculated as the time from addition of benzaldehyde until 50% of the cells had divided. The broken line represents the median cell-cycle duration of NHIK 3025 cells treated with benzaldehyde in the same way as described above. These data were presented previously [3] and are here presented together with the protein data in order to compare the inhibition of the two growth parameters: cell-cycle time and protein-doubling time.

It has been reported that certain aliphatic aldehydes are able to reduce the protein synthesis of bone marrow, liver and hepatoma cells grown *in vitro* as primary cultures [5–7]. Perin *et al.* [5] reported data indicating the L-erythro-α,β-dihydroxybutyraldehyde reduced the leucine incorporation into protein of primary cultures when present in concentrations above about 1 mM. Similarly, although our techniques as well as cell type were different from those of Perin *et al.*, we find that benzaldehyde inhibits protein synthesis of NHIK 3025 cells for about the same drug concentrations: >0.5 mM (Fig. 4).

In a more recent paper Watanuki and Sakaguchi [19] reported that benzaldehyde inhibited the uptake of both 2-deoxy-D-glucose and various nucleosides into SV-40-transformed rat embryo fibroblasts (W-3Y-23), while little or no effect was found with respect to incorporation of amino acids. However, the highest concentration of benzaldehyde studied was 50 μg/ml (~0.5 mM) which, according to our results, would be too low to affect the protein synthesis significantly. Thus the present results are not in contradiction to those of Watanuki and Sakaguchi.

The present results further show that inhibition of protein synthesis by benzaldehyde results in a reduction in the rate of accumulation of protein (see Figs 1 and 2). Simultaneously, the cell-cycle duration is also increased to such an extent that it is about equal to the protein doubling time (see Fig. 4). Thus, even in the presence of benzaldehyde, there is a balance between the rate of cell-cycle progression and the rate of protein accumulation. This finding is similar to that reported earlier on cells of the same line exposed to the specific protein synthesis inhibitor cycloheximide (<1.25 μM) [4, 20]. In both cases the rate of cell-cycle progression and protein accumulation is reduced by the same factor in the presence of the drug. The cells might therefore be in balanced growth in the presence of these protein synthesis inhibitors and might even be able to grow at a reduced rate for an unlimited time. Since it is well established that specific inhibition of cell-cycle progression, e.g. with DNA-synthesis inhibitors, does not necessarily result in a reduced accumulation of protein [21, 22], it is not probable that the cell-cycle inhibition induced by benzaldehyde (as well as cycloheximide [4]) during interphase is the primary growth parameter inhibited by the drugs. Rather, it is likely that both drugs primarily inhibit the protein accumulation and that the rate of cell-cycle progression is reduced secondarily.

There is, however, one difference in cell-cycle inhibition between the two drugs: benzaldehyde induces a marked prolongation of mitosis [3, 23]. Since such an effect is not seen with cycloheximide (unpublished observation), the mitotic inhibition is not an effect which generally follows when the rate of protein synthesis or protein accumulation is reduced. Taking into account that the cells both in the presence of cycloheximide and in the presence of benzaldehyde double their protein content before they enter mitosis, there are two alternative explanations for the difference in action between cycloheximide and benzaldehyde: (i) the mitotic inhibition is a result of other effects of benzaldehyde than those leading to inhibition of protein synthesis; or (ii) the mitotic inhibition and the inhibition of protein synthesis are both secondary effects induced by benzaldehyde inhibiting a specific type of biochemical process.

The first alternative leaves many unknown possibilities for molecular mechanisms leading to mitotic inhibition. It is, however, striking to observe that the extra inhibition of mitosis compared with interphase takes place at about the same concentration of benzaldehyde that induces a severe inhibition of the protein synthesis (~3 mM) [3]. This makes it more probable that both the mitotic inhibition and the inhibition of protein synthesis results from similar primary effects. Thus alternative (ii) should not be ruled out.

On the basis of the type of antitumour effect that has been reported with benzaldehyde, alternative (ii) is of particular hypothetical interest. Kochi *et al.* [2] reported that the tumour tissue re-differentiates after long-term treatment with this drug. This de-transformation of the cells could result if benzaldehyde was in some way able to reduce the activity of special growth-regulatory proteins. Such proteins, e.g. pp60c-src [24], while necessary in small quantities for regulation of cell growth, may be able to induce transformation when present in a greater amount, as may happen if the genes from which they are coded (oncogenes) are over-activated. By reducing the functionality of such proteins, e.g. by reducing the rate of phosphorylation or some other activation process, benzaldehyde might specifically reduce the transforming ability of the oncogene products. If the proteins necessary for protein synthesis as well as the proteins necessary for completing mitosis (e.g. tubulin) have to be activated by a mechanism similar to that of the growth-regulatory and transforming oncogene products, all 3 effects (the inhibition of protein synthesis, the mitotic inhibition and the de-transformation of malignant cells) could be natural consequences of the same type of primary effect induced by benzaldehyde: the inhibition of a process which activates enzymes.

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