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HYDROGEN PEROXIDE AND THE PROLIFERATION OF BHK-21 CELLS

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Intracellular levels of H_2O_2 in BHK-21 cells are not static but decline progressively with cell growth. Exposure of cells to inhibitors of catalase, or glutathione peroxidase, not only diminishes this decline but also depresses rates of cell proliferation, suggesting important growth regulatory roles for those antioxidant enzymes. Other agents which also diminish the growth-associated decline in intracellular levels of H_2O_2, such as the superoxide dismutase mimic, copper II – (3,5-diisopropylsalicylate)$_2$, or docosahexaenoic acid, also reduced cell proliferation. In contrast, proliferation can be stimulated by the addition of 1 μM exogenous H_2O_2 to the culture medium. Under these conditions, however, intracellular levels of H_2O_2 are unaffected, whereas there is a reduction in intracellular levels of glutathione. It is argued that critical balances between intracellular levels of both H_2O_2 and glutathione are of significance in relation both to growth stimulation and inhibition. In addition growth stimulatory concentrations of H_2O_2, whilst initially leading to increased intracellular levels of lipid peroxidation breakdown products, appear to "trigger" their metabolism, possibly through aldehyde dehydrogenase, whose activity is also stimulated by H_2O_2.

KEY WORDS: BHK-cells, cell proliferation, superoxide, hydrogen peroxide, glutathione peroxidase, catalase, lipid aldehydes, aldehyde dehydrogenase.

INTRODUCTION

There have been a number of observations to suggest that cellularly generated superoxide, or hydrogen peroxide, might act as novel cellular "messengers" capable of promoting growth responses in cultured mammalian cells[1–3]. A wide variety of normal and malignant cell types are known to generate and release superoxide and hydrogen peroxide either in response to cytokine/growth factor stimulus or constitutively in the case of tumour cells[2,3]. Other studies serve to indicate intracellular sources of superoxide generation[3–6]. The growth promoting mechanisms could involve direct interaction with specific receptors, or oxidation of growth signal transduction molecules such as growth factor receptors[7], protein kinases[8,9], protein phosphatases[10], transcription factors[11–15] or transcription factor inhibitors[3,15]. Whilst a specific and direct interaction with any protein remains to be demonstrated it is nevertheless possible that hydrogen peroxide might modulate the redox state and activity of these important signal transduction proteins indirectly through changes in cellular levels of reduced and oxidised glutathione.[3,14–16]

In a previous study we examined intracellular levels of reduced glutathione (GSH) in relation to the growth of baby hamster kidney fibroblasts (BHK-21/C13)[16]. Intracellular levels of GSH were not static but declined progressively as the growth rate of these cells increased. On the other hand, exposure of these cells to mercaptosuccinate, an inhibitor of glutathione peroxidase, not only reduced the rate of this decline but also retarded cell proliferation. A similar effect was observed after exposure of BHK cells

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to high concentrations of N-acetylcysteine and suggested a special relationship between cell proliferation and cellular GSH levels in these cells.

In light of this potential role of glutathione peroxidase in the modulation of cell proliferation, we have now explored the possible importance of intracellular hydrogen peroxide in relation to BHK-cell growth. Like those of GSH, intracellular levels of hydrogen peroxide were also not static but declined with increasing BHK-cell proliferation. It was nevertheless possible to arrest this decline by treatment of the cells with inhibitors of glutathione peroxidase or catalase, which also brought about depressed proliferation.

Additional studies showed that in general, growth inhibition of BHK cells occurred, either (a) when the decline of ***both*** hydrogen peroxide and GSH is diminished, or (b) when there is a severe depletion of one or other of GSH or hydrogen peroxide. In contrast, growth stimulation of BHK-21 cells by 1 μM hydrogen peroxide surprisingly did not actually affect the overall cellular levels of hydrogen peroxide, although a modest decline in cellular levels of GSH has been observed previously[16] under these conditions. An additional effect of 1 μM hydrogen peroxide was the apparent triggering of increased cellular metabolism of growth-inhibitory aldehydic lipid peroxidation breakdown products.

EXPERIMENTAL PROCEDURES

Cell culture

Baby hamster kidney fibroblasts (BHK-21/C13) were grown in monolayer cultures in 3.5 cm petri-dishes in 2 ml Eagle's minimal essential medium supplemented with 10% (v/v) calf serum (Gibco BRL, Paisley) and proliferation assessed as previously described.[16]

Determination of hydrogen peroxide

In order to determine levels of H_2O_2 in BHK-21 cells a method based on that of Okuda *et al.*[17] was used. The medium was first removed from the monolayer cultures. The monolayers were then washed two times with phosphate buffered saline (PBS). 2 ml 0.2 M perchloric acid were then added and the suspension containing precipitated cellular material was transferred to centrifuge tubes and centrifuged at 2,500 g for 3 minutes at 4°C. The supernatant was removed and neutralised to pH 7.5 with 5 M KOH. This was then centrifuged 10 000 g for 2 minutes at 4°C. 200 μl of the supernatant was applied to a 1 ml column of anion exchange resin (AG 1-x2, Bio Rad) and the column washed with 800 μl distilled water and the eluate used for the determination of H_2O_2 as outlined by Okuda *et al.*[17] Reaction mixtures contained 1 ml eluate, 400 μl 12.5 mM 3-dimethylaminobenzoic acid (DMAB) in 0.375 M phosphate buffer pH 6.5, 80 μl 0.01M 3-methyl-2-benzothiazoline hydrazone (MBTH) and 20 μl horse radish peroxidase (17 units/ml). After 3 minutes at 20°C the absorbance of the solution was determined at 590 nm and compared with increases elicited by standard samples of hydrogen peroxide.

To determine the levels of H_2O_2 in the medium, the medium (2 ml) was removed and treated with 150 μl 40% (w/v) trichloroacetic acid. The precipitated proteins were removed by centrifugation at 2,500 g for 3 minutes at 4°C. The supernatant was removed and neutralised to pH 7.5 with 5 M NaOH. This was then centrifuged at

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10,000 g for 2 minutes at 4°C and 1 ml samples used for assay of H_2O_2 using DMAB, MBTH and horse radish peroxidase as above.

This method has previously been used to detect intracellular hydrogen peroxide generation in cold stressed plants.[17] However because it employs an initial acid-extraction of the BHK-21 cells the hydrogen peroxide detected could arise as a result of artefactual release from cellular biomolecules such as haem proteins under the initial acid conditions. However this is likely to be insignificant. In initial attempts to detect intracellular hydrogen peroxide, BHK cells were first homogenised in dilute phosphate buffer before the addition of acid. This however led to negligible levels of detectable hydrogen peroxide, presumably due to the extensive metabolism of hydrogen peroxide by cellular catalase and glutathione peroxidase during the time-consuming homogenisation procedure. Nevertheless if significant levels of hydrogen peroxide (similar to those shown in Figure 1) were artefactually generated by acid treatment of the biomolecules remaining in the homogenised cells, they would have been readily obvious in this earlier procedure. Additionally when the method is applied to calf serum, which contains low levels of haemoglobin, significant levels of hydrogen peroxide are only detected after the serum has been in contact with BHK-21 cells (see Figure 6).

Determination of lipid peroxidation breakdown products

The medium was removed from the cultured BHK-21 monolayers and the cells washed two times with PBS (phosphate buffered saline). The monolayers were then scraped off into 2 ml PBS and the cells collected by centrifugation at 4°C for 2 minutes at 2,500 g. The cells were then lysed in 0.5 ml 20 mM tris-HCI pH 7.4. The lysate was then centrifuged at 2,500 g for 8 minutes at 4°C. 200 μl samples of the supernatant recovered from this low-speed centrifugation were used for assay of decomposition products of peroxidised polyunsaturated fatty acids (i.e. malonaldehyde and 4-hydroxyalkenals) using the LPO-586 colorimetric assay from Bioxytech S.A., France, following their instructions.

Determination of aldehyde dehydrogenase activity

For assay of aldehyde dehydrogenase, cell monolayers were washed two times with 60 mM potassium phosphate buffer pH 7.4 and then harvested by scraping off into 1 ml of 5 mM sodium pyrophosphate buffer pH 9.6 and disrupted by sonication on ice for 60 secs in 5 sec bursts and then centrifuged at 12 000 g for 5 mins. The supernatant was then used for enzyme assay following the procedure of Kraemer and Deitrich.[18] Reaction mixtures (1 ml) contained 50 mM sodium pyrophosphate, 6 mM NAD, 1 mM propionaldehyde and activity was assayed by following increase in absorbance at 340 nM.

Reagents

N-acetylcysteine, mercaptosuccinate, docosahexaenoic acid, horse radish peroxidase, aminotriazole, DMAB and MBTH were obtained from Sigma Chemical Co. Copper II (3,5-diisopropylsalicylate)$_2$ was from Aldrich Chemical Co.

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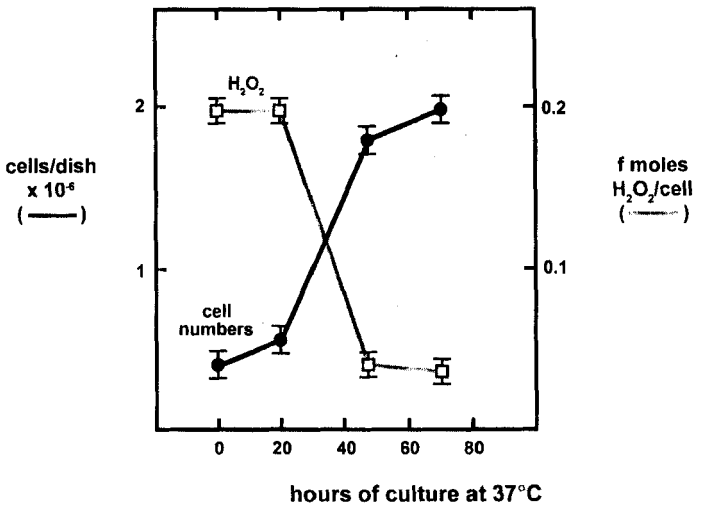

FIGURE 1 Cellular levels of hydrogen peroxide in relation to BHK-21 cell growth. Triplicate 3.5 cm petri-dishes were seeded with 0.29×10^6 BHK-21 cells in 2 ml growth medium which were allowed to grow at 37°C. At the times indicated, cultures were removed for determination of live cell numbers (●) and cellular levels of H_2O_2 (□) as described in EXPERIMENTAL PROCEDURES. Results are presented as the means of triplicate experiments ± s.d. (n = 3).

RESULTS

Previously we have shown that as growth of BHK-21/C13 cells (baby hamster fibroblasts) progresses, intracellular levels of reduced glutathione (GSH) decline[7]. Figure 1 shows that intracellular levels of hydrogen peroxide also decline with increasing growth.

On the basis that hydrogen peroxide within animal cells would be expected to be metabolised either by catalase or glutathione peroxidase, the effect of inhibitors of these enzymes on the growth related decline in intracellular hydrogen peroxide was investigated. Table 1 shows that both aminotriazole (inhibitor of catalase) and mercaptosuccinate (inhibitor of glutathione peroxidase[19]) diminished the growth related decline in intracellular hydrogen peroxide. Significantly both enzyme inhibitors depressed proliferation of BHK-21/C13 cells (Table 1).

In view of this apparent link between cellular hydrogen peroxide levels and cell proliferation the effect of other agents which might be expected to influence cellular hydrogen peroxide levels were explored. Exposure of cells to copper II (3,5-diisopropylsalicylate)$_2$, (CuDIPS), a low molecular weight lipophilic mimic of superoxide dismutase also decreased the rate of cellular hydrogen peroxide decline as well as impairing cell proliferation (Figure 2). The same was true (Figure 2) for cells exposed to the n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA), which we have previously found to increase the rate of intracellular superoxide generation in BHK-21 cells.[20] Another means of increasing intracellular superoxide generation in these cells is to withdraw serum[4,6]. This also leads to less growth. Again it can be seen from Figure 3, the decline in intracellular levels of hydrogen peroxide is slower in cells grown under these conditions.

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TABLE 1

Effects of aminotriazole and mercaptosuccinate on BHK-21 cell proliferation and intracellular levels of hydrogen peroxide.

(a) Additions	Cells/plate $\times 10^{-6} \pm$ s.d. (n = 3) at various times after additions					
				24 hr	48 hr	72 hr
none	0.97 ± 0.04	1.93 ± 0.02	2.30 ± 0.03			
aminotriazole (150 μM)	0.70 ± 0.02	1.42 ± 0.03	1.85 ± 0.03			
mercaptosuccinate (150 μM)		0.73 ± 0.03	1.33 ± 0.03	1.84 ± 0.01		
(b) Additions	at various times after additions			f moles/cell intracellular $H_2O_2 \pm$ s.d. (n = 3)		
				24 hr	48 hr	72 hr
none	0.36 ± 0.02	0.15 ± 0.04	0.16 ± 0.01			
aminotriazole (150 μM)	0.49 ± 0.04	0.28 ± 0.07	0.26 ± 0.01			
mercaptosuccinate (150 μM)		0.67 ± 0.05	0.47 ± 0.01	0.33 ± 0.02		

Triplicate petri-dishes (3.5 cm) were seeded with 0.6×10^6 BHK-21 cells in 2 ml growth medium. After 24 hr of growth at 37°C, aminotriazole or mercaptosuccinate was added to certain cultures as indicated. At various times following these additions, live cell numbers and cellular levels of H_2O_2 were determined as in EXPERIMENTAL PROCEDURES. Results are presented as the means of triplicate experiments ± s.d. (n = 3).

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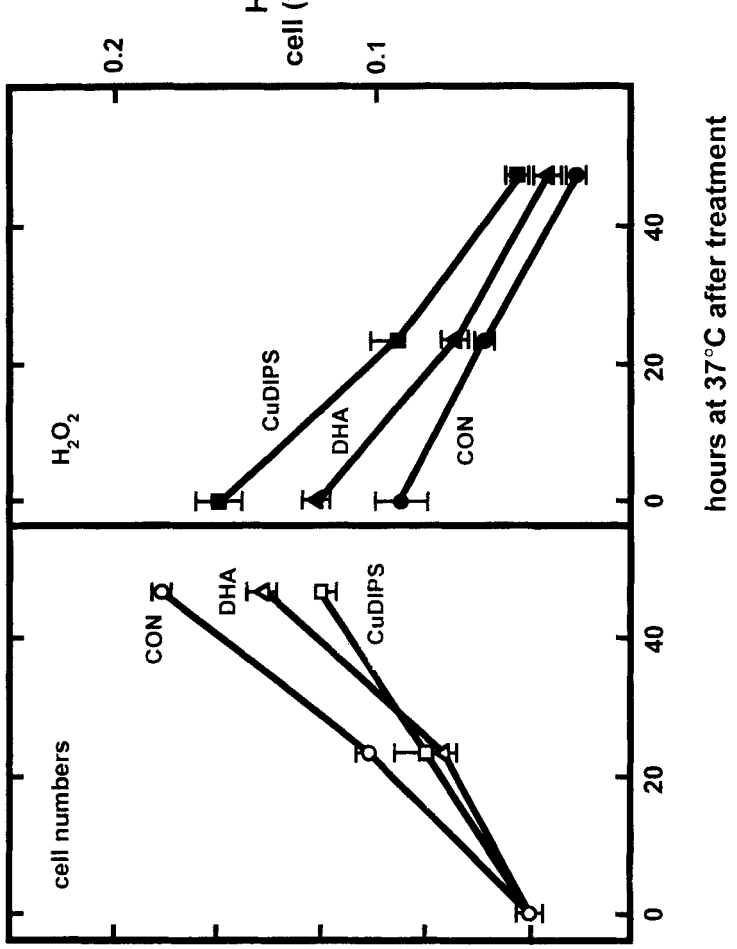

FIGURE 2 The effects of CuDIPS and docosahexaenoic acid on BHK-21 cell growth and cellular levels of hydrogen peroxide. Triplicate 3.5 cm petri-dishes were seeded with 0.14×10^6 BHK-21 cells in 2 ml growth medium which were allowed to grow for 24 hr at 37°C. At that time certain cultures were treated with 10 μM copper II (3,5-diisopropylsalicylate)$_2$ (CuDIPS) in 1% ethanol or 100 μM docosahexaenoic acid (DHA) in 1% ethanol. Control cultures were treated with 1% ethanol. At the times indicated thereafter live cell numbers and cellular levels of H_2O_2 were determined as described in EXPERIMENTAL PROCEDURES. Cell numbers; control cultures (○); cultures treated with CuDIPS (□), or DHA (△). H_2O_2 levels; control cultures (●), cultures treated with CuDIPS (■) or DHA (▲) Results are presented as means of triplicate experiments ± s.d. (n = 3).

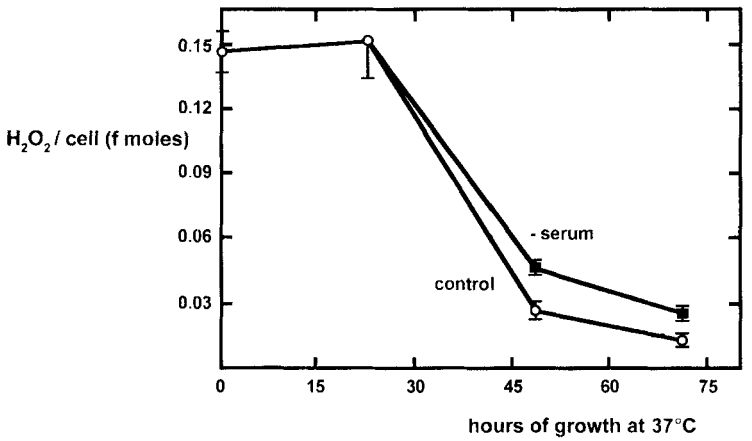

FIGURE 3 The effect of serum withdrawal on cellular levels of H_2O_2 in BHK-21 cells. Triplicate 3.5 cm petri-dishes were seeded with 0.29×10^6 BHK-21 cells in 2 ml growth medium which were allowed to grow at 37°C, but after 24 hr the medium in certain cultures was replaced with medium lacking serum. At the times indicated intracellular levels of H_2O_2 were determined as for Figure 1. Control cultures (O), serum deprived cultures (■). Results are presented as the means of triplicate experiments ± s.d. (n = 3).

The ability of these reagents to diminish the rate of cellular hydrogen peroxide decline contrasts sharply with the effects of N-acetylcysteine (NAC). Exposure of BHK-21 cells to 10 mM NAC not only causes a depression of cell proliferation, it also reduces levels of cellular H_2O_2 to below those detectable by the technique used (Figure 4).

Another possible approach to influencing the levels of cellular hydrogen peroxide is exposure of cells to hydrogen peroxide added exogenously to the medium (Figure 5). Past experience[16] showed that whilst the addition of 1 μM H_2O_2 was growth stimulatory to BHK-21 cells, 100 μM H_2O_2 was growth inhibitory[16]. When the effects of such additions were assessed in terms of intracellular hydrogen peroxide levels, it appeared (surprisingly) that addition of 1 μM hydrogen peroxide to the medium did not actually have a significant effect on intracellular cellular levels, nor the rate of their decline with growth (Figure 5). Such data should be considered in conjunction with the information in Figure 6 which shows the *actual* media concentrations measured following the exogenous addition of hydrogen peroxide to the culture plates. Although hydrogen peroxide was added in the culture medium at either 1 μM or 100 μM, it was nevertheless metabolised very rapidly (even in the time taken to collect the media for assay!) to leave the media concentrations indicated (Figure 6).

Another cell parameter that appears to decline with growth of BHK-21 cells is the intracellular level of breakdown products of lipid peroxidation, such as malonaldehyde and 4-hydroxyalkenals (Figure 7). Whilst such breakdown products are likely to arise from peroxidised membrane lipids it should be stressed that their intracellular levels are not indicative of rates of cellular lipid peroxidation. Moreover the cellular processes leading to such lipid peroxidation are not clear. Possibly they involve cellularly generated hydrogen peroxide or superoxide. Perhaps significantly, exposure of BHK-21 cells to exogenous hydrogen peroxide (either 1 μM or 100 μM) rapidly leads to elevated cellular levels of these lipid peroxidation breakdown products (again even in the time taken to collect the cells for assay) (Figure 8). However, in the ensuing 24 hr of growth, those relatively high levels in the hydrogen peroxide treated cells declined

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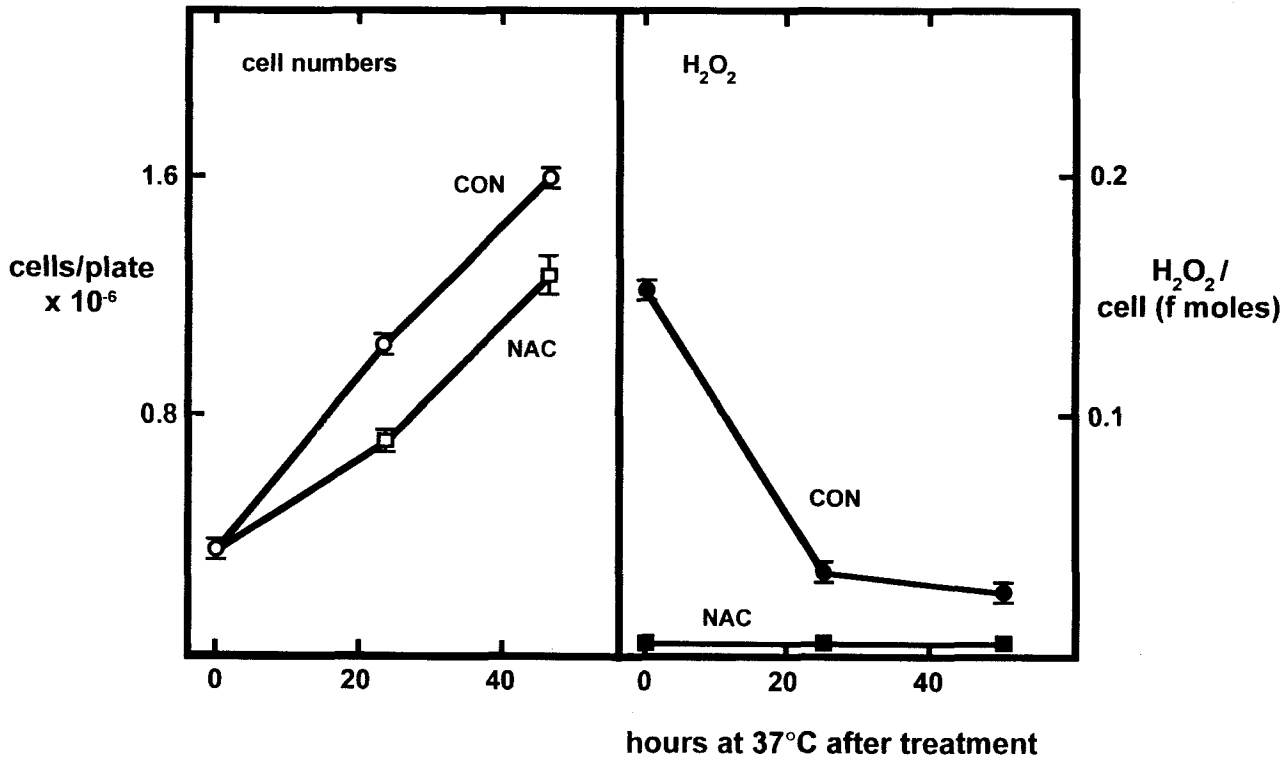

FIGURE 4 The effect of N-acetylcysteine on BHK-21 cell growth and cellular levels of H_2O_2.Triplicate 3.5 cm petri-dishes were seeded with 0.20×10^6 BHK-21 cells in 2 ml growth medium which were allowed to grow for 24 hr at 37°C. At that time some cultures were treated with 10 mM N-acetylcysteine (NAC). At the times indicated thereafter live cell numbers and cellular hydrogen peroxide levels were assessed as in Figure 1. Cell numbers; control cells (○), NAC-treated cells (❑). Cellular H_2O_2; control cells (●), NAC-treated cells (■). Results are presented as the means of triplicate experiments ± s.d. (n = 3).

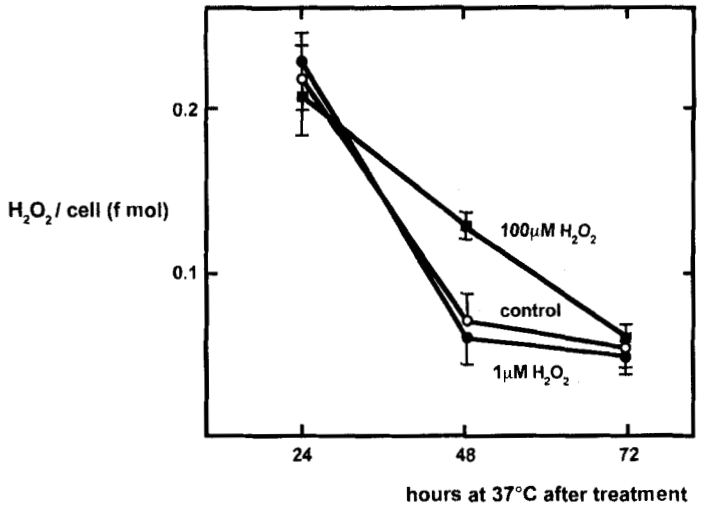

FIGURE 5 The effects of exposure to exogenous H_2O_2 on cellular levels of H_2O_2 in BHK-21 cells. Triplicate 3.5 cm petri-dishes were seeded with $0.18 \times 10^{6+}$ BHK-21 cells in 2 ml growth medium which were allowed to grow at 37°C for 24 hr. At that time various cultures were exposed to single doses of exogenous H_2O_2 at 1 μM and 100 μM. At the times thereafter intracellular H_2O_2 levels were determined as described in Figure 1, in control cells (○); in cells exposed to 1 μM H_2O_2 (●); in cells exposed to 100 μM H_2O_2 (■). Results are presented as the means of triplicate experiments ± s.d. (n = 3).

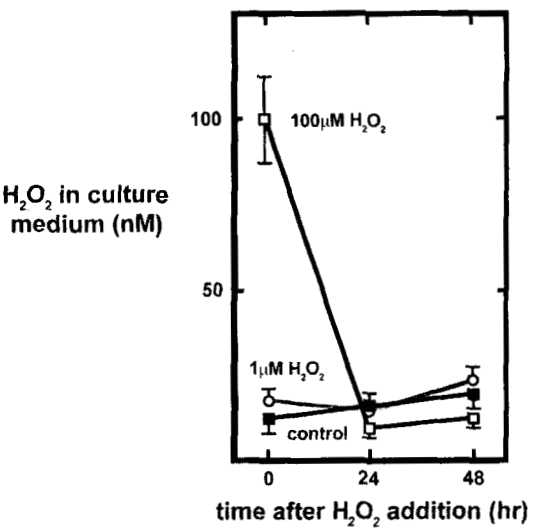

FIGURE 6 Concentrations of hydrogen peroxide in the culture medium following addition of exogenous hydrogen proxide to cultures of BHK-21 cells. Triplicate monolayer cultures of BHK-21 cells were established (0.35×10^6 cells per 3.5 cm petri-dish in 2 ml gwth medium). After 24 hr of growth at 37°C, H_2O_2 was added to the final concentrations indicated. Growth was then allowed to continue and the medium removed from cultures at the times indicated for he determination of the *remaining* concentration of H_2O_2 as detailed in EXPERIMENTAL PROCEDURES. Media concentrations of H_2O_2 in cultures of untreated cells (■); in cultures of cells treated with 1 μM H_2O_2 (○) or 100 μM H_2O_2 (□). Resus are expressed as the means of triplicate experiments ± s.d. (n = 3).

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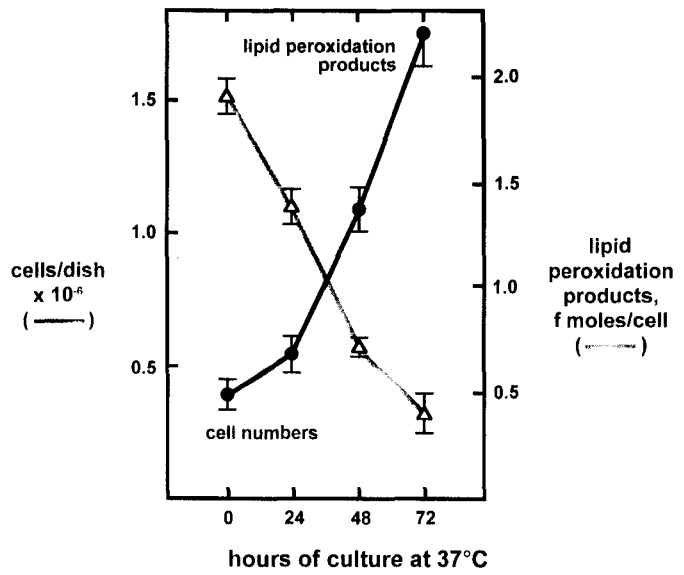

FIGURE 7 Lipid hydroperoxide breakdown products and BHK-21 cell proliferation. Triplicate 3.5 cm petri-dishes were seeded with 0.38×10^6 BHK-21 cells in 2 ml growth medium and allowed to grow at 37°C. At the times indicated cultures were removed for determination of live cell numbers (●) and cellular levels of lipid hydroperoxide breakdown products (△) as described in EXPERIMENTAL PROCEDURES. Results are expressed as the means of triplicate experiments ± s.d. (n = 3).

at rates actually greater than those encountered in control cells (Figure 8). Thereafter levels of lipid peroxidation breakdown products were similar to those encountered in control cells.

Since the intracellular levels of lipid peroxidation products could be regulated by the activity of metabolising enzymes such as aldehyde dehydrogenase[21], and/or glutathione transferase[22], the activity of these enzymes was explored. Whilst the level of glutathione transferase activity in BHK-21 cells was negligible, as is the case for most permanently cultured cell lines, overall levels of aldehyde dehydrogenase activity, whilst detectable, declined somewhat with growth (Figure 9). Nevertheless the cellular level of aldehyde dehydrogenase could be stimulated by prior exposure of cells to hydrogen peroxide added at 1 μM (Figure 9). In a separate experiment direct addition of 1 μM hydrogen peroxide for 15 min at 37°C to cell extracts prepared from previously untreated cells led to the stimulation of aldehyde dehydrogenase activity in these extracts by 81%. Whether this is indicative of a direct effect on the enzyme of course cannot be decided.

DISCUSSION

As was the case for cellular levels of GSH in BHK-21 cells[16], cellular levels of hydrogen peroxide in these cells were not static, but declined progressively with growth. Previous work of ours suggested that cellularly generated hydrogen peroxide[1,3] may be important in growth regulation. This is strengthened by the observations that interference with the enzymes catalase and glutathione peroxidase, that would normally metabolise

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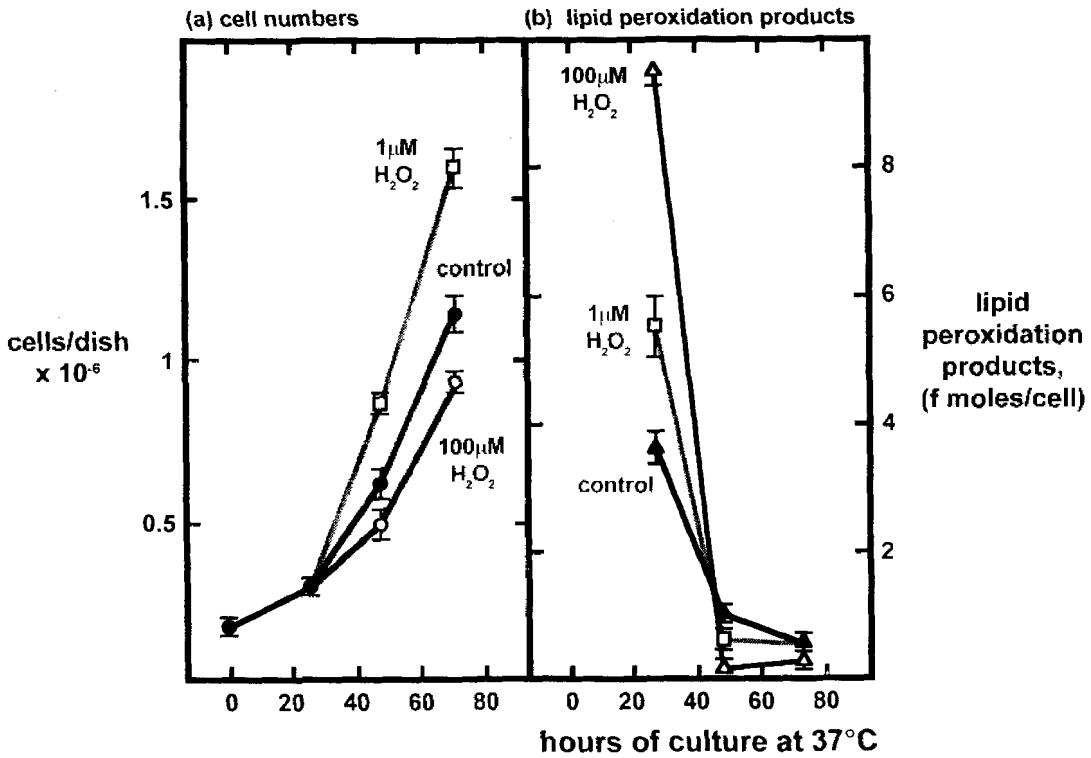

FIGURE 8 The effect of exogenous hydrogen peroxide on BHK-21 cell growth and the levels of lipid peroxidation breakdown products. Triplicate monolayer cultures of BHK-21 cells were established ($0.15 \times$ cells per 3.5 cm petri-dish in 2 ml growth medium). After 24 hr of growth at 37°C, H_2O_2 was added as indicated to final concentrations of 1 μM or 100 μM. Growth was then allowed to continue and cultures were removed for determination in (a) of live cell numbers in untreated control cultures (●), in cells treated with 1 μM H_2O_2 (○), and in (b) cellular levels of lipid hydroperoxide breakdown products in untreated control cells (▲); in cells treated with 1 μM H_2O_2 (❑); in cells treated with 100 μM H_2O_2 (Δ). Results are expressed as the means of triplicate experiments ± s.d. (n = 3).

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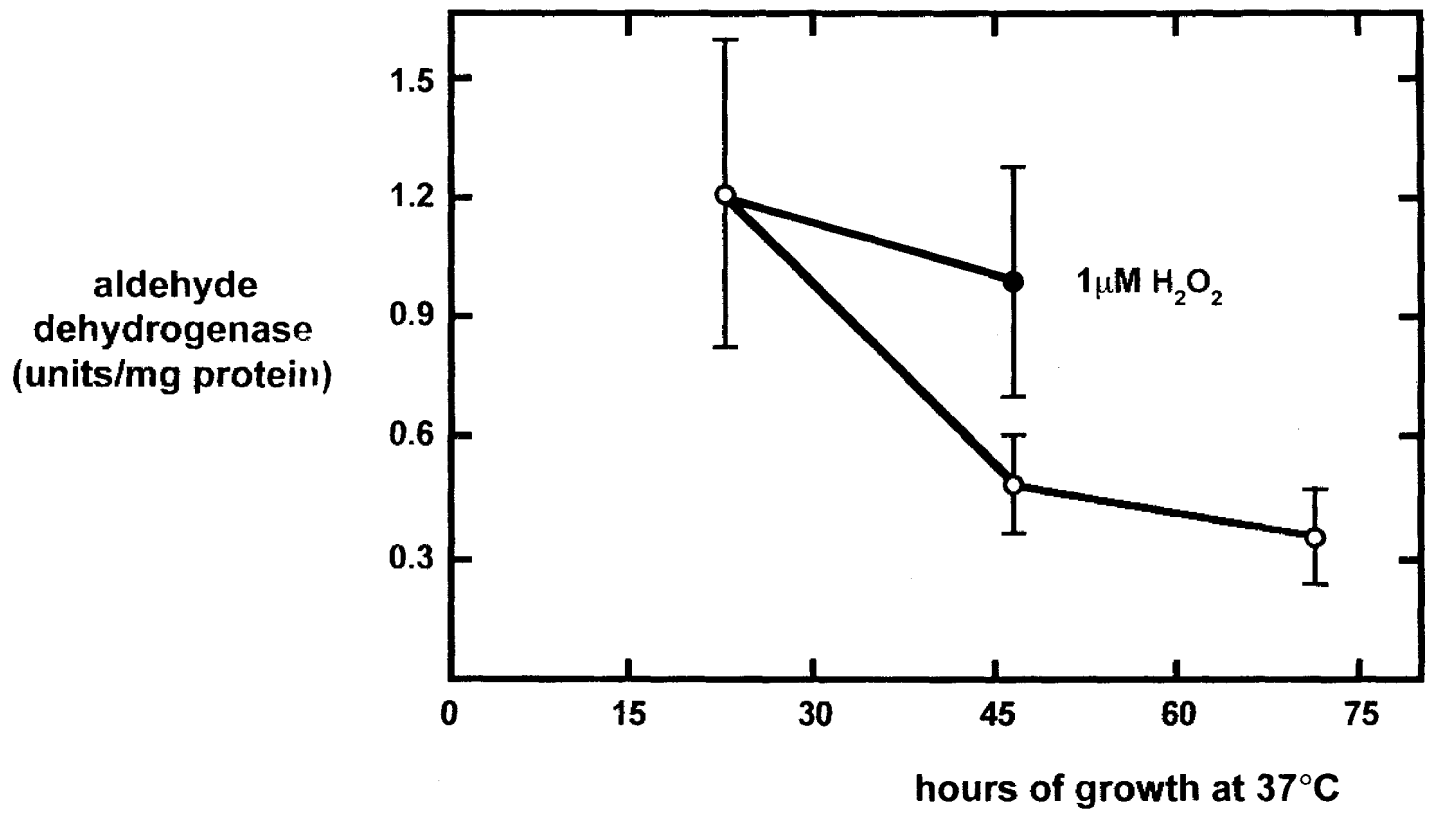

FIGURE 9 Aldehyde dehydrogenase activity in BHK-21 cells. Aldehyde dehydrogenase activity (○) was determined in extracts prepared from BHK-21 cells at various times of growth after initial seeding into petri-dishes, as described in EXPERIMENTAL PROCEDURES. In certain cases cultures were taken after 24 hr of growth at 37°C and H_2O_2 added to 1 μM and growth continued for 24 hr before assay for aldehyde dehydrogenase (●). Results are expressed as the means of determinations on extracts from triplicate experiments ± s.d. (n = 3). Enzyme activity is in arbitrary units.

hydrogen peroxide, not only diminished the hydrogen peroxide decline but also reduced BHK cell proliferation. Other agents that would be expected to increase cellular levels of hydrogen peroxide, such as cell permeable superoxide dismutase mimics or docosahexaenoic acid, had similar effects. Furthermore, exposure of BHK cells to growth inhibiting levels of exogenous hydrogen peroxide also diminished the growth associated decline of intracellular hydrogen peroxide.

Despite the view that hydrogen peroxide can cross cell membranes it may be significant that our procedures reveal considerable residual levels of intracellular hydrogen peroxide despite removal of the growth medium and washing of the cell monolayers. It is, of course, a feature of eukaryotic cells that there are many intracellular compartments which may have reduced accessability to the washing procedure. Some organelles themselves of course may be involved in hydrogen peroxide generation such as mitochondria[4]. Another possibility, for which there is as yet no data, is that certain cellular proteins may specifically have a high affinity for hydrogen peroxide (e.g. putative receptor type proteins).

In a previous study we demonstrated that inhibition of glutathione peroxidase diminished the rate of GSH decline during BHK-cell growth. Thus in those circumstances when cell growth is repressed, the normal decline of ***both*** hydrogen peroxide ***and*** GSH is diminished. This is also true of BHK-21 cells deprived of serum. Not only is the decline in cellular hydrogen peroxide reduced (Figure 3) but also the rate of GSH decline is less (see reference 16). However, in other situations such exposure of cells to growth inhibitory levels of exogenous hydrogen peroxide (i.e. 100 μM) whilst the rate of intracellular hydrogen peroxide decline is reduced, we previously demonstrated that the intracellular level of GSH declines very significantly (reference 16). In contrast, when cell proliferation is inhibited by 10 μmM NAC, whilst the rate of GSH decline is diminished[16], there is an almost complete loss of cellular hydrogen peroxide (Figure 4).

The progressive alterations in cellular levels of GSH and hydrogen peroxide with growth may reflect a kinetic situation. Different cellular levels of GSH and H_2O_2 may be required depending on different growth rates, where distinctly different activities of growth signal transduction proteins may be necessary. Overall it appears that growth of BHK-21 cells is reduced either (a), under conditions where the progressive decline in ***both*** intracellular GSH and hydrogen peroxide is retarded or (b), under conditions where intracellular levels of GSH ***or*** hydrogen peroxide are severely reduced, possibly resulting in cellular conditions that are either overly "oxidative" or too "reductive" for optimal growth. Such proposals emphasise critical balances in cellular H_2O_2 and GSH levels and the possible function of glutathione peroxidase, catalase and superoxide dismutase as novel growth regulatory molecules. In this particular context it may be significant that when growth stimulatory concentrations of hydrogen peroxide (i.e. 1 μM) are added to cultures of BHK-21 cells, cellular levels of hydrogen peroxide and their rate of decline are surprisingly unaffected (Figure 5). However, our previous study[16] showed that under such conditions there is a slight increase in the rate of decline of cellular GSH[7]. Thus exogenously hydrogen peroxide at 1 μM may stimulate growth through effects primarily on cellular GSH concentrations rather than those of hydrogen peroxide *per se*. It is suggested that this could involve the participation of glutathione peroxidase.

Whilst intracellular levels of hydrogen peroxide and GSH may be critical in growth control a possible "trade-off" in terms of this novel signalling system may be peroxidative membrane alterations. In terms of cell proliferation control, lipid peroxidation could have a number of "down-regulatory" effects. Most of the proteins that play key

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roles in proliferative signal transduction function in a membrane environment, or in close association with membranes, and it is well-established that the activity of integral membrane proteins can be modulated by the lipids of the bilayer[23]. Lipid peroxides can also break down non-enzymically to yield a variety of carbonyls, such as the hydroxyalkenals. These aldehydes, and in particular 4-hydroxynonenal (HNE), can react with thiol and amino groups of nearby proteins affecting several enzymic activities[24]. These effects, however, appear to occur at HNE concentrations greater than 10 μM. At low "non-toxic" concentrations other effects have been observed which may have considerable relevance to cell proliferation[25]. These include the stimulation of adenyl cyclase activity in liver membranes[26] and an inhibition of ornithine decarboxylase activity[25], and the modulation of globin gene and prooncogene *c-myc* expression in K562 murine leukaemia cells[27–28].

Some analyses have shown that lipid peroxidation and the concentration of its breakdown products are relatively low in undifferentiated highly proliferating tumour cells[29,30], and it has been hypothesised that products of lipid peroxidation such as HNE may play a central role in the "down-regulation" of cell proliferation. The physiological levels of HNE have been found to range 0.2 to 2.8 μM. These levels represent a "steady-state" level of HNE because it is continuously produced and rapidly catabolised by normal cells[31].

Although several studies, including our own[32,33], have indicated low levels of lipid peroxidation in normal mammalian cells that are rapidly dividing, the studies in this report show that cellular levels of lipid peroxidation breakdown products can nevertheless vary widely in the same cell line simply depending on the state of growth. Initially in cultures of BHK-21 cells when growth is minimal, levels of lipid peroxidation breakdown products (malonaldehyde and 4-hydroxyalkenals) are relatively high but these decline as growth rate increases (Figure 7). Events leading to the oxidation of cellular lipids in BHK-21 cells, however, remain to be elucidated. Nevertheless, the experiments in which exogenously added H_2O_2 increase cellular levels of lipid peroxidation breakdown products, suggest that cellularly generated H_2O_2 could be involved somehow.

Until recently it had been assumed that the levels of lipid aldehydes measured in cells reflected their level of production. However, it is now recognised that these represent steady-state levels resulting from the balance between their production and metabolism[31]. With regard to the decline in detectable breakdown products of lipid peroxidation that appears to be associated with BHK-cell proliferation, there are a number of possibilities. For instance the MDA and 4-hydroxyalkenals measured could be metabolised to an increased extent. 4-Hydroxyalkenal has been suggested as a cellular substrate of gluthatione transferase[22] but another cellular enzyme that may be significant in this metabolic context is aldehyde dehydrogenase[21]. It appears that class 1 and class 2 aldehyde dehydrogenases oxidise malonaldehyde whereas the oxidation of 4-hydroxyalkenals requires the activity of class 3 aldehyde dehydrogenases[21]. In the case of BHK-21 cells there was little detectable glutathione transferase and although aldehyde dehydrogenase activity was detectable this nevertheless decreased with growth. However, a notable observation was that exposure of cells to 1 μM hydrogen peroxide was sufficient to stimulate overall aldehyde dehydrogenase activity over a 24 hr period (Figure 9). Thus amongst its growth related effects, 1 μM hydrogen peroxide may stimulate the aldehyde dehydrogenase catalysed oxidation of aldehydic lipid peroxide breakdown products and thus reduce their growth limiting effects. Whatever the explanation, perhaps the most surprising observation is that an apparent "trigger" to increased cell proliferation and loss of lipid peroxidation breakdown

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products can be achieved by a single "bolus" of 1 μM H_2O_2 added to the culture medium (Figure 8), which is nevertheless metabolised extremely rapidly (Figure 6).

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