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Research Article

The effect of hydrogen peroxide on the cyclin D expression in fibroblasts

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Abstract. Activation of mitogen-activated protein (MAP) kinase is essential for cyclin D1 expression and provides a link between mitogenic signalling and cell cycle progression. Hydrogen peroxide (H_2O_2) activates MAP kinase; however, it is not known whether this leads to cyclin D expression. Sustained expression of cyclin D1 and D2 was observed when Her14 fibroblasts were incubated with 3 mM or higher H_2O_2 concentrations. Similar results were obtained when cells were incubated in the presence of serum (FCS). However, the sustained expression of cyclin D1 and D2 upon H_2O_2 treatment was not due to the MAP kinase pathway, because MAP kinase kinase inhibitors did not inhibit cyclin D expression. Furthermore, cyclin D1 and D2 levels remained constant even after addition of a protein synthesis inhibitor, indicating that the effect of H_2O_2 was not due to induction of protein synthesis. These results indicate that H_2O_2 reversibly inhibits the ubiquitin-proteasome dependent degradation of cyclin D1 and D2, probably by transiently inhibiting ubiquitination and/or the proteasome.

Key words. Hydrogen peroxide; oxidative stress; cyclin D; MAP kinase; signal transduction; ubiquitin-proteasome pathway.

Introduction

Hydrogen peroxide (H_2O_2), a reactive oxygen species produced endogenously during several physiological processes, is able to induce a number of events via oxidative modifications, which are also induced by mitogens. After H_2O_2 treatment, proteins involved in signal transduction pathways become activated, including protein kinase C (PKC) [1, 2], epidermal growth factor (EGF) receptor (EGFR) [3, 4], and mitogen-activated protein (MAP) kinases [5–7].

The effects of H_2O_2 on the EGF-induced signal transduction pathway have been studied in particular detail [3]. H_2O_2 increases the phosphorylation of the EGFR [8], and H_2O_2-induced tyrosine phosphorylated EGFR forms a complex with SHC-Grb2-SOS followed by the activation of Ras and the extracellular-signal-regulated protein kinases (Erks) [3]. Furthermore, H_2O_2 activates protein kinase B (PKB) via an EGFR/phosphatidylinositol 3 (PI 3) kinase pathway [9]. The mechanism involved in the increased phosphorylation of the above-mentioned proteins by H_2O_2 is not currently clear, however, there are some indications that it could be due to inhibition of phosphatases [10]. Moreover, we have demonstrated recently that H_2O_2 inhibits EGFR internalization in fibroblasts [11].

During normal regulation of the cell cycle, early activation of the MAP kinase pathway initiates events leading to induction of cyclin D expression and subsequent progression through the cell cycle [12]. The expression of activated Ras is associated with the increased expression of cyclin D1 [13] in both epithelial cells [14] and fibroblasts [15]. Furthermore, in the absence of growth factors, ac-

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tivation of the Raf1-MKK-p42/p44MAP kinase pathway by a constitutively active Raf-1 mutant is sufficient to induce cyclin D1 transcription [16]. At later points in the progression through the G1 phase, other pathways may continue to provide stimulatory signals to maintain cyclin D expression. Some of the proteins involved are Ral-GDS, PI 3 kinase, PKB, and Rac [17, 18]. Taking the above points into consideration one would expect H_2O_2 to have an effect on the proteins involved in the regulation of cell cycle progression, because continuous activation of the EGFR and MAP kinase, induced by H_2O_2, would lead to continuous induction of cyclin D1. The purpose of this study was to determine whether cyclin D expression is influenced by H_2O_2.

We show sustained expression of cyclin D1 and D2 when Her14 fibroblasts were incubated with different H_2O_2 concentrations. Our results indicate that this effect is due to inhibition of the ubiquitin-proteasome-dependent degradation of cyclin D1 and D2.

Materials and methods

Materials

For the incubations the following chemicals were used: cyclohexamide (Sigma, Zwijndrecht, The Netherlands), U0126 (Promega, Madisin, Wis.), PD98059 and carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG-132) (both from Biomol Research Laboratories, Plymouth, Pa.).

Cell culture

Her14 cells (NIH-3T3 mouse fibroblasts stably transfected with cDNA encoding human EGFR) were maintained in Dulbecco's modified eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 7.5% foetal calf serum (FCS, Gibco) in a humidified 5% CO_2 atmosphere at 37 °C at a final density of approximately 6×10^4 cells/cm^2. Cells at passages 5–25 were used for experiments. When cells were incubated at 37 °C in the absence of CO_2 for several hours, DMEM-Hepes (DMEM buffered with 25 mM Hepes) supplemented with 7.5% FCS was used. Phosphate-buffered saline supplemented with 5 mM glucose (PBS_{gluc}) is required as the dilution medium for H_2O_2, rather than DMEM-Hepes, since components of the latter react with H_2O_2 [19] to alter its concentration.

Lactate dehydrogenase release

The fibroblasts were incubated with different concentrations of H_2O_2 for 1 or 2 h. The incubation medium was collected and 0.1% Triton X-100 in PBS was added to the cells for 1 h. After lysis, samples were collected once more. Subsequently, an excess of catalase (100–1000 units/ml incubation fluid; Sigma) was added to all the samples to degrade H_2O_2, preventing H_2O_2 from reacting with pyruvate, which is a substrate in the subsequent lactate dehydrogenase (LDH) assay. LDH activity was measured by the decrease in absorbance at 340 nm, due to the conversion of NADH to NAD by LDH in the presence of pyruvate, as described previously [20].

Western blotting

At the end of the incubation, cells (6×10^4 cells/cm^2) were rinsed twice in ice-cold PBS and then lysed in RIPA buffer containing 20 mM Tris/HCl pH 7.4, 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM benzamidine, 1 mM PMSF and 250 µM Na_3VO_4 at 4 °C for 10 min (60 µl RIPA/9.62-cm^2 plate). The lysates were clarified by centrifugation at 10,000 × g for 5 min at 4 °C. The supernatant was transferred to another tube and 13 µl was used to measure the protein concentration with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). To the supernatant, 20 µl of 4× sample buffer (33% glycerol, 0.3 M DTT, 6.7% SDS, 0.01% bromphenol blue, 80 mM Tris/HCl pH 6.8) was added. The samples were boiled for 10 min and 20 µg of each sample was separated in 10–12% SDS-polyacrylamide gels and transferred into polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim, Almere, The Netherlands) by standard procedures. Immunodetection was performed at room temperature by first blocking the PVDF membrane for 1 h in PBS containing 0.05% Tween 20 (PBS-T) and 2% milk powder. This was followed by incubation with diluted antiserum in the washing buffer PBS-T containing 0.5% milk powder for 1 h. The antisera used were as follows: rabbit anti-cyclin D1/D2 (Upstate Biotechnology, Lake Placid, N.Y.), rabbit anti-phosphorylated p42/p44^{MAPK} (New England Biolabs, Beverly, Mass.) and mouse anti-myosin (Biotrend Chemikalie, Cologne, Germany) as loading control. The membranes were then washed three times and incubated for 1 h with horseradish peroxidase-conjugated donkey anti-mouse (DAM-PO) or goat anti-rabbit immunoglobulin G (GAR-PO) (Jackson ImmunoResearch Laboratories, West Grove, Pa.), diluted 1:5000 in the washing buffer containing 0.5% milk powder. After washing, immune complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer's specifications (NEN Life Science, Boston, Mass.).

Results

Regulation of cyclin D expression by serum factors

Activation of the MAP kinase pathway by growth factors initiates events leading to induction of cyclin D expression [12]. Figure 1a shows a significant decrease in cyclin D1 and D2 when growing Her14 fibroblasts are incubated in serum-free DMEM-Hepes for 2, 3 or 5 h. This correlates with a decrease in the phosphorylation of MAP kinase. When FCS is added to the medium, sustained in-

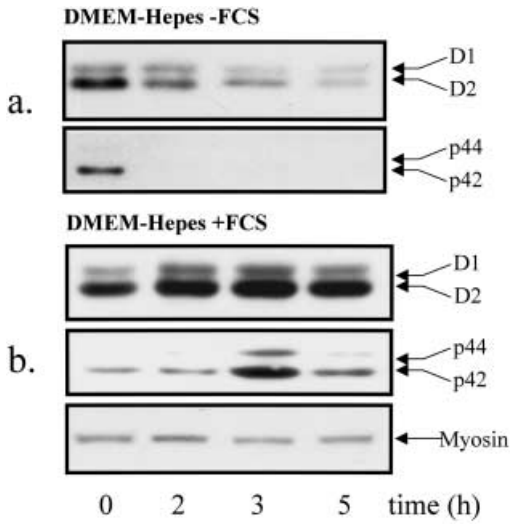

Figure 1. Regulation of cyclin D expression by FCS in DMEM-Hepes. Her14 cells were incubated for 2, 3 or 5 h in serum-free DMEM buffered with Hepes (DMEM-Hepes – FCS) in the absence (*a*) or presence (*b*) of FCS. Cyclin D1 (36 kDa), D2 (34 kDa) protein levels and phosphorylation of MAP kinases (p42/p44) were determined by Western blot analysis (see Materials and methods). Myosin (210 kDa) was used as a loading control for all the blots. A representative blot of myosin is shown in the lower panel of *b*. Experiments were repeated at least three times and similar results were obtained: representative blots are shown.

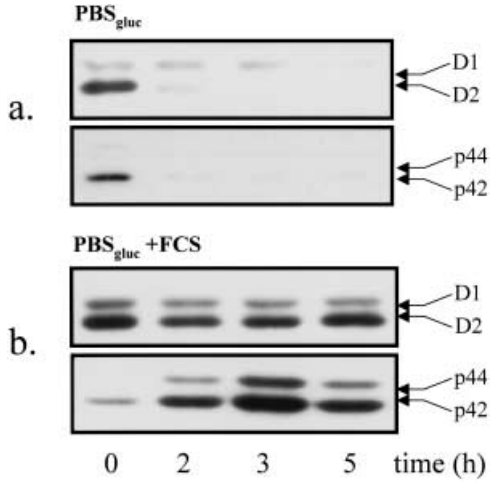

Figure 2. Regulation of cyclin D expression by FCS in PBS$_{gluc}$ medium. Her14 cells were incubated for 2, 3 and 5 h in PBS$_{gluc}$ medium in the absence (*a*) or presence (*b*) of FCS. Cyclin D1 (36 kDa) and D2 (34 kDa) protein levels and phophorylation of MAP kinases (p42/p44) were determined by Western blot analysis (see Materials and methods). Experiments were repeated at least three times and similar results were obtained: representative blots are shown.

duction of cyclin D1 and D2 is observed (fig. 1b), correlating with a transient activation of MAP kinase (fig. 1b). This transient activation of MAP kinase is due to addition of medium with fresh FCS. These results demonstrate that FCS-induced signals play a crucial role in the expression of D-type cyclins, apparently through MAP kinase.

Effect of H_2O_2 on cyclin D expression

Since several components of DMEM-Hepes, among them pyruvate, react with H_2O_2 and lower the H_2O_2 concentration to unknown levels [19], PBS$_{gluc}$ was used to incubate the cells with H_2O_2. When Her14 fibroblasts were incubated in PBS$_{gluc}$ without H_2O_2, cyclin D1 and D2 decreased rapidly (fig. 2a), correlating with dephosphorylation of MAP kinase (fig. 2a). However, in the presence of FCS, cyclin D1 and D2 levels did not diminish (fig. 2b) and MAP kinase was transiently phosphorylated (fig. 2b), as also observed in DMEM-Hepes. These results show that the different media used do not affect the FCS-induced signals towards cyclin D expression. In previous studies, we showed that FCS leads to the activation of MAP kinase and induction of cyclin D1 [12], and that H_2O_2 is able to activate MAP kinase by inducing its phosphorylation [5]. Other groups have also reported the activation of MAP kinase by H_2O_2 [6, 7]. However, whether H_2O_2 can also induce the expression of cyclin D it not known. Therefore, Her14 fibroblasts were incubated in PBS$_{gluc}$ in the presence or absence of 3 mM H_2O_2 for various periods of time. This concentration of H_2O_2 was chosen after performing of a dose-response experiment (fig. 3a). In the absence of H_2O_2, a decrease in cyclin D1 and D2 protein levels was observed (fig. 4a), while in the presence of H_2O_2, cyclin D1 and D2 protein levels remained sustained (fig. 4b).

H_2O_2 toxicity for cells depends on its concentration, incubation time and the cell type. To test the toxicity of different H_2O_2 concentrations on this cell type, the integrity of the cells was examined. As shown in figure 5, even in the presence of 5 mM H_2O_2, no LDH release was measured, clearly showing that no significant cell lysis occurred. The cells can also be treated with PBS$_{gluc}$ for 4 h without losing their integrity (data not shown).

The role of MAP kinase

To establish whether the effect of H_2O_2 on cyclin D expression is due to activation of MAP kinase, as found for growth-factor-induced cyclin D expression, Her14 cells were incubated with H_2O_2 in the presence of MAP kinase kinase (MKK) inhibitors, U0126 [21] or PD98059 [22]. MKK is able to phosphorylate p42/p44 MAP kinase. U0126 inhibits the kinase activity of MKK, but not its phosphorylation, while PD98059 inhibits the phosphorylation of MKK by Raf. Our experiments were performed with growing cells (non-serum-starved cells) and MAP kinase is therefore partly phosphorylated in control cells (fig. 3). In other studies, activation of MAP kinase has been observed when serum-starved cells are treated with H_2O_2 [5–7]. Her14 cells were treated with PBS$_{gluc}$ in the absence or presence of different concentrations of

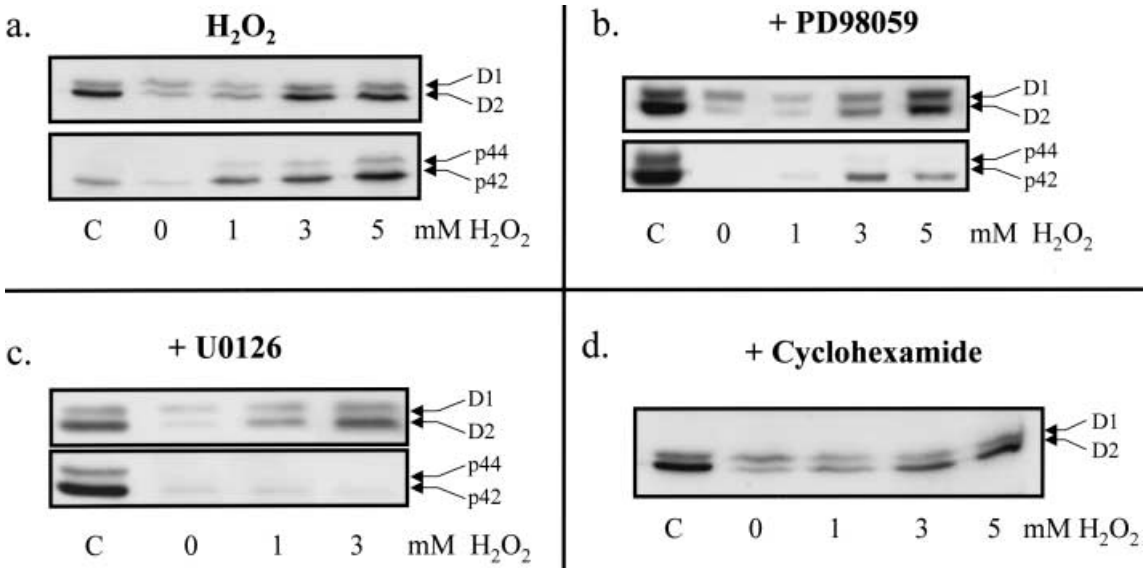

Figure 3. Effect of MAP kinase kinase (MKK) and protein synthesis inhibitors on cyclin D expression. Control cultures (C) were untreated. Experiments were repeated at least three times and similar results were obtained: representative blot is shown. (*a*) Her14 fibroblasts were incubated with PBS_{gluc} in the absence or presence of different concentrations of H_2O_2 for 2 h. Cyclin D1 (36 kDa) and D2 (34 kDa) and MAP kinase-P protein levels were determined by Western blot analysis. (*b*) PBS_{gluc} with 50 µM PD98059 was used for 2 h in the presence or absence of 1, 3 and 5 mM H_2O_2. (*c*) Her14 cells were exposed to PBS_{gluc} with 50 µM U0126 for 2 h in the presence or absence of 1 and 3 mM H_2O_2. (*d*) Her14 cells were treated with PBS_{gluc} containing 25 µM cyclohexamide in the presence or absence of 1, 3 and 5 mM H_2O_2 for 2 h.

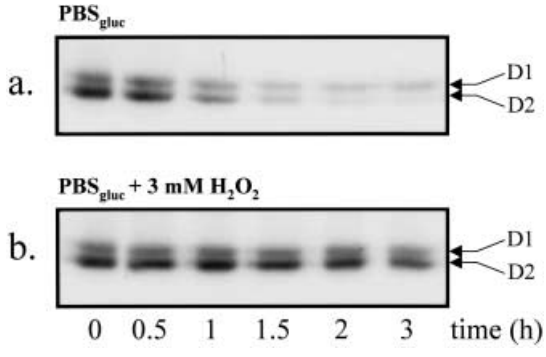

Figure 4. Regulation of cyclin D expression by H_2O_2. Her14 fibroblasts were incubated in PBS_{gluc} in the absence (*a*) or presence (*b*) of 3 mM H_2O_2 for the times indicated. Cyclin D1 (36 kDa) and D2 (34 kDa) protein levels were determined by Western blot analysis (see Materials and methods). Experiments were repeated at least three times and similar results were obtained: representative blots are shown.

H_2O_2. As shown in figure 3a, in the presence of 3 and 5 mM H_2O_2, cyclin D1 and D2 expression remained sustained and MAP kinase was phosphorylated. These results suggest a correlation between the phosphorylation of MAP kinase and cyclin D expression, when the cells are exposed to 3 and 5 mM H_2O_2. Surprisingly, in some experiments when cells were incubated with 1 mM H_2O_2, MAP kinase phosphorylation increased but cyclin D1 and D2 levels diminished. To investigate whether the phosphorylation of MAP kinase was involved in the effect of H_2O_2 on cyclin D1 and D2 expression, the following experiments were performed. First, the cells were treated with 50 µM PD98059 diluted in PBS_{gluc} in the absence or presence of varying H_2O_2 concentrations for 2 h. After the incubation with 3 and 5 mM H_2O_2, partial dephosphorylation of MAP kinase was detected (fig. 3b); however, cyclin D1 and D2 levels did not significantly decrease (fig. 3b) in comparison to the PBS_{gluc} incubation. Second, to eliminate the possibility that the partial phosphorylation of MAP kinase in the presence of PD98059 was responsible for the cyclin D expression, Her14 fibroblasts were exposed to 50 µM U0126 in PBS_{gluc} in the absence or presence of 1 or 3 mM H_2O_2 for 2 h. Figure 3c shows that MAP kinase was completely dephosphorylated after the incubation with U0126. This inhibitor prevents the phosphorylation of MAP kinase by H_2O_2. However, U0126 had no effect on cyclin D1 and D2 expression (fig. 3c). These observations suggest that the effect of H_2O_2 on cyclin D1 and D2 expression does not involve the activation of MAP kinase.

Effect of inhibition of protein synthesis

We subsequently investigated whether protein synthesis is required for the sustained presence of cyclin D1 and D2 in the presence of H_2O_2. Experiments were performed in PBS_{gluc} in the presence of 25 µM cyclohexamide and different H_2O_2 concentrations. Figure 3d shows that sustained expression of cyclin D1 and D2, when incubated in the presence of 3 or 5 mM H_2O_2, is not affected by cyclo-

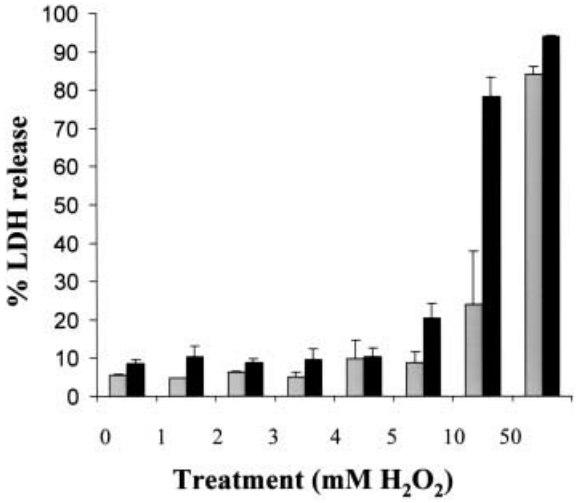

Figure 5. Effect of H_2O_2 on LDH release. Her14 cells were treated with PBS_{gluc} in the absence or presence of 1, 3, 4, 5, 10 and 50 mM H_2O_2 for 1 (grey bars) or 2 (solid bars) h as indicated. LDH release was measured as described in Materials and methods. Values are expressed as the mean ± SD (n = 3).

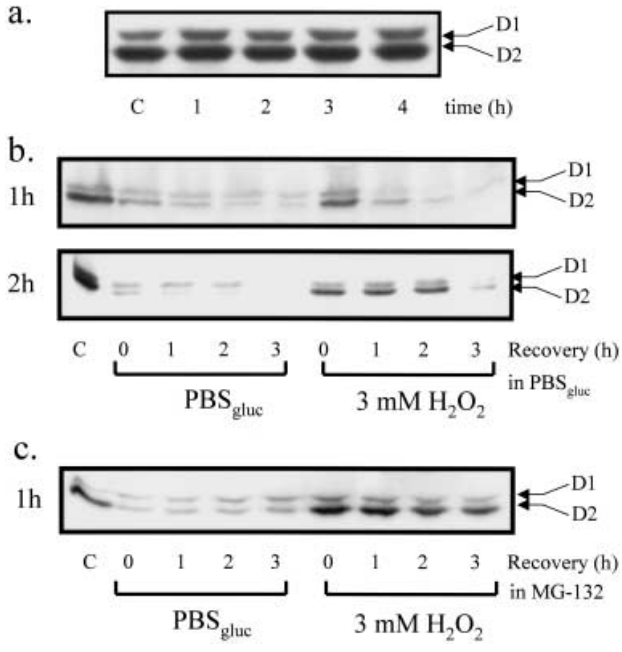

Figure 6. Regulation of cyclin D expression by a proteasome inhibitor and the effect of H_2O_2 removal. Control cultures (C) were untreated. (*a*) Her14 cells were treated with PBS_{gluc} in the presence of 25 µM MG-132 for 1, 2, 3 and 4 h. (*b*) Her14 cells were exposed to PBS_{gluc} in the presence or absence of 3 mM H_2O_2 for 1 h or 2 h. Thereafter, the cells were incubated in PBS_{gluc} for various periods of time as indicated. (c) Her14 cells were incubated in PBS_{gluc} in the presence or absence of 3 mM H_2O_2 for 1 h. Thereafter, the cells were incubated in PBS_{gluc} in the presence of 25 mM MG-132 for 1, 2 or 3 h.

hexamide. These results indicate that the effect of H_2O_2 on cyclin D1 and D2 levels is not due to an induction of cyclin D synthesis, suggesting that the effect is through inhibition of the degradation of these cyclins.

The effect of proteasome-dependent protein degradation inhibition

D-type cyclins are degraded through the ubiquitin-proteasome pathway [23–25]. To establish whether degradation of cyclin D1 and D2 indeed occurs through this pathway in Her14 cells, PBS_{gluc} was added to the cells in the presence or absence of 25 µM of the proteasome inhibitor MG-132 [26]. With the proteasome inhibitor, cyclin D1 and D2 protein levels remained constant (fig. 6a). In the absence of MG-132, degradation of cyclin D1 and D2 occurred as shown before (fig. 2a). These observations confirm that cyclin D1 and D2 are degraded through the ubiquitin-proteasome pathway.

Reversibility of the H_2O_2 effect

Our results demonstrate that cyclin D1 and D2 are degraded through the ubiquitin-proteasome pathway and that H_2O_2 inhibits cyclin D1 and D2 degradation, suggesting that it inhibits the ubiquitin-proteasome pathway. Inhibition of ubiquitination [27, 28] and the 26S proteasome [29] has been reported after H_2O_2 treatment in various cell types. Both effects are known to be reversible. Therefore, we studied the reversibility of cyclin D degradation after H_2O_2 treatment. Cells were exposed to PBS_{gluc} in the absence or presence of 3 mM H_2O_2 for 1 or 2 h and subsequently allowed to recover in PBS_{gluc} for various times. Figure 6b shows that cyclin D was degraded after 1 h of recovery in PBS_{gluc}, when the cells were exposed for 1 h to 3 mM H_2O_2. After 2 h treatment, the cells required 3 h in PBS_{gluc} for cyclin D degradation. When the cells were recovered in the presence of MG-132 after 1 h of 3 mM H_2O_2, cyclin D degradation did not occur (fig. 6c). Similar results were found after 2 h of 3 mM H_2O_2 (data not shown). These results demonstrate that H_2O_2 reversibly inhibits the ubiquitin-proteasome-dependent degradation of cyclin D1 and D2.

Discussion

The focus of this study was to investigate the effect of H_2O_2 on the expression of cyclin D1 and D2. H_2O_2 is able to activate MAP kinase [5–7], and this protein is involved in the induction of cyclin D1 expression [13–16]. In both media, DMEM-Hepes and PBS_{gluc}, growth factors are required to activate MAP kinase and to induce cyclin D1 and D2 expression. Sustained expression of cyclin D1 and D2 was observed when Her14 fibroblasts were incubated in PBS_{gluc} containing 3 mM H_2O_2. Furthermore, when cells were exposed to H_2O_2, phosphorylation of

MAP kinase was detected. However, this did not correlate with the sustained cyclin D1 and D2 expression, since after addition of the MKK inhibitors, U0126 and PD98059, MAP kinase was dephosphorylated, but cyclin D1 and D2 levels did not decrease. These observations demonstrate that the sustained expression of cyclin D1 and D2 is not mediated through the phosphorylation of MAP kinase by H_2O_2. However, the present experiments cannot exclude that downstream members of the MAP kinase pathway may be a target of H_2O_2.

To investigate whether protein synthesis was involved in this effect, cyclohexamide, a protein synthesis inhibitor, was used. In the presence of cyclohexamide, cyclin D1 and D2 levels remained constant upon H_2O_2 treatment, indicating that the effect of H_2O_2 was not caused by an induction of cyclin D synthesis. Since the level of D-type cyclins is determined by the balance between rates of synthesis and degradation, H_2O_2 may be able to inhibit one or more mechanism(s) of cyclin degradation.

Cyclins D1 and D2 become degraded through the ubiquitin-proteasome pathway. Linkage of ubiquitin to the cyclin is a highly organized process involving the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-ligase (E3). Poly-ubiquitinated proteins are degraded by the 26S proteasome [30]. Components of an E3 ubiquitin-ligase complex, the SKP1/Cullin/F-box (SCF) protein complex, discovered in yeast and conserved in humans [31], may be responsible for the degradation of G1/S cyclins. Cyclin D1 and D2 share a common mechanism of degradation, and they associate with Cul-1, a component of the SCF complex [25]. Several groups have reported that H_2O_2 is able to inhibit ubiquitin and the proteasome in their systems [27–29, 32, 33]. The molecular mechanism underlying inhibition of the ubiquitin-proteasome system can occur at two levels: the ubiquitinating enzymes and/or the proteasome itself may become inhibited.

In cultured bovine lens epithelial cells, an increase in endogenous ubiquitin conjugates is detected in the response to oxidative stress along with a diminished ability to form conjugates using exogenous ubiquitin [27, 28]. In intact retina and retinal pigment epithelial cell models exposed to H_2O_2, the levels of multi-ubiquitinated proteins decrease and the levels of free ubiquitin increase. These results are consistent with the decrease in E1 and E2 activity. This effect is reversible in less than 1 h after removal of H_2O_2 [32]. A comparable sequence of events has been observed for the EGFR [33]. Alternatively, H_2O_2 is able to inactivate ATP-stimulated 26S proteasomal proteolysis, and thus the ubiquitinated proteins cannot be degraded [29]. At the moment, we cannot discriminate between the two possible mechanisms underlying inhibition of the ubiquitin-proteasome system in the present study. However, the system(s) is clearly only transiently inhibited, since upon removal of the oxidative stress, cyclin D1 and D2 are rapidly degraded.

In conclusion, the present study demonstrates that sustained expression of cyclin D1 and D2 after H_2O_2 treatment is not caused by the induction or activation of proteins like MAP kinase. These results suggest that H_2O_2 reversibly inhibits the ubiquitin-proteasome-dependent degradation of cyclin D, probably by inhibiting ubiquitination and/or the proteasome.

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