H$_2$O$_2$ Induces a Transient Multi-phase Cell Cycle Arrest in Mouse Fibroblasts through Modulating Cyclin D and p21$^{Cip1}$ Expression*

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To defend against the potential damages induced by reactive oxygen species, proliferating cells enter a transient cell cycle arrest. We treated mouse fibroblasts with H$_2$O$_2$ and found that sublethal doses of H$_2$O$_2$ induce a transient multi-phase cell cycle arrest at the G$_1$, S, and G$_2$ phases but not the M phase. Western blot analysis demonstrated that this transient cell cycle arrest is associated with the down-regulation of cyclins D1 and D3 and up-regulation of the CKI p21$^{Cip1}$ expression. We also demonstrated that the induction in p21$^{Cip1}$ expression by H$_2$O$_2$ is at least partially mediated at the transcriptional level and can occur in the absence of p53 function. Further immunoprecipitation kinase and immunodepletion assays indicated that in response to H$_2$O$_2$ treatment, the down-regulation of cyclin Ds expression is associated with repression of cyclin D-CDK4, whereas the accumulation of p21$^{Cip1}$ is responsible for the inhibition of cyclin E and A-CDK2 activity and associated with the down-regulation of cyclin B-CDC2 activity. These data could account for the cell cycle arrest at the G$_1$, S, and G$_2$ phases following H$_2$O$_2$ stimulation. Deletion of p21$^{Cip1}$, restoration of cyclin D expression, or overexpression of cyclin E alone is insufficient to effectively overcome the cell cycle arrest caused by sublethal doses of H$_2$O$_2$. By contrast, overexpression of the human Herpesvirus 8 K cyclin, which can mimic the function of cyclin D and E, is enough to override this transient cell cycle arrest. On the basis of our findings, we propose a model in which moderate levels of H$_2$O$_2$ induce a transient multi-phase cell cycle arrest at least partially through up-regulation of p21$^{Cip1}$ and down-regulation of cyclin D expression.

Reactive oxygen species (ROS),$^1$ including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (H$^\cdot$O) are natural by-products generated by living organisms as a consequence of aerobic metabolism. (1–3). Accumulation of excess ROS, which are toxic to cells, can cause oxidative stress leading to damage to proteins, nucleic acid, and cell membranes (4, 5). Oxidative stress has also been implicated in a variety of human diseases, including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and aging (2, 3, 6). To protect against the potentially detrimental effects caused by elevated levels of ROS, cells deploy antioxidant defenses and activate damage repair and replacement systems. To counter oxidative stress, cells constitutively express enzymes that neutralize ROS and repair and replace the damage caused by ROS (4, 5). In addition, cells also mount “adaptive responses” to elevated levels of oxidative stress. Mammalian cells respond to oxidative stress with an increase in expression of antioxidant enzymes, including glutathione S-transferases, peroxidases, and superoxide dismutases, and activation of protective genes, including those encoding the heat shock proteins. Stationary phase (noncycling) cells are intrinsically resistant to high levels of ROS, whereas actively dividing cells are prone to oxidative damage, because their DNA is uncoiled and exposed while they engage in rapid DNA replication. To defend against the potential damages induced by oxidative stress, proliferating cells enter a transient cell cycle arrest, during which DNA is protected by histone proteins, energy is conserved through reduced expression of non-essential genes, and the expression of shock and stress proteins is increased. This transient growth arrest also allows time to repair and/or replace the damaged DNA so that the mutated DNA will not be replicated and/or transferred to daughter cells. This also gives the cells extra time to mount an adaptive response to counteract further oxidative damage. Thus, this transient growth arrest is a crucial component of the cellular response to oxidative stress (7, 8).

However, besides transient cell cycle arrest, the cell also exhibits a wide range of adaptive cellular responses ranging from transient growth arrest, to permanent growth arrest, to

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$^1$ The abbreviations used are: ROS, reactive oxygen species; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; MEF, mouse embryonic fibroblast; IPTG, isopropyl-β-D-thiogalactoside; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; PCNA, proliferating cell nuclear antigen; FACS, fluorescence-activated cell sorting.
apoptosis, and ultimately to necrosis, depending on the level of oxidative stress experienced (7, 8). Sublethal levels of ROS induce a temporary cell cycle arrest that is believed to protect cells from DNA damage itself, consuming excess energy and resources and incorporating mutations following DNA damages. Following repair or detoxification, the dividing cells reinitiate cell cycle progression. However, when the oxidative stress is too severe and the cells do not have the ability to adapt or resist the stress or to repair the damaged cellular components, the cells may respond by undergoing permanent cell cycle arrest or apoptosis. At even higher levels of ROS, cells undergo cell death by necrosis. Nevertheless, recent emerging evidence also demonstrates that ROS are physiological mediators of cell signaling and functions and are produced by a variety of cells after stimulation with cytokines, peptide growth factors, and agonists of receptors (9–11). For example, cytosolic ROS produced in response to stimulation by growth factors are involved in mediating the proliferative response (11). Therefore, depending on the level, ROS exerts two physiological effects: damage to various cellular components and activation of specific signaling pathways.

The mammalian cell division cycle is traditionally divided into G1 (gap phase 1), S (DNA synthesis), G2 (gap phase 2), and M (mitosis) phases. Progression through each phase of the cell cycle is controlled by co-operative activity of distinct cyclin-dependent kinases (CDKs) and their regulatory subunits, cyclins (12). The cyclins have specificity for different CDK subunits. The D-type cyclins (cyclins D1, D2, and D3) bind to and activate CDK4 and CDK6 preferentially, whereas cyclin E interacts predominantly with CDK2, cyclin A associates primarily with CDK2 and CDC2 (12–14), and cyclin B associates specifically with CDC2 (also called CDK1) (12, 15, 16). The association of CDK4 or CDK6 with D-type cyclins is important for G1 phase progression, whereas the CDK2-cyclin E complex is essential for initiation of the S phase. Progression through the S phase is regulated by the CDK2-cyclin A complex, whereas the transition from G2 to M is mediated by CDC2-cyclin B. The principal cellular substrates of the cyclin-CDKs are members of the retinoblastoma protein (pRB) family of pocket proteins (pRB, p107, and p130) (17–19). In their hypophosphorylated forms, these pocket proteins bind to members of the E2F family of transcription factors, thereby negatively regulating transcription of E2F-dependent genes that are required for entry into and transition through S phase of the cell cycle (17, 20–22). During G1 to S transition, phosphorylation of pRB is initiated by cyclin D-dependent kinases and is completed by cyclin E-CDK2 and cyclin A-CDK2 (12, 13). CDKs are negatively regulated by two classes of CDK inhibitors (CKIs): the CIP/KIP and the INK4 families of proteins. The CIP/KIP proteins (p21\(^{Cip1}\), p27\(^{Kip1}\), and p57\(^{Kip2}\)) target both cyclin D-CDK4/6 and cyclin E/CDK2 by binding to the cyclin-CDK complexes, whereas the INK4 family (p16\(^{INK4a}\), p15\(^{INK4b}\), and p18\(^{INK4c}\)) specifically inhibit cyclin D-CDK4/6 complexes through direct association with the CDK components, thereby preventing their interaction with D-type cyclins (12, 13). The CIP/KIP proteins are inhibitors of cyclin E- and A-dependent CDK2, but p21\(^{Cip1}\) (more than p27\(^{Kip1}\)) at low stoichiometric levels acts as positive regulator of cyclin D-dependent CDK4/6 kinases (23, 24).

An understanding of the cellular responses to oxidative stress will provide useful insights into the mechanisms of aging and transformation as well as the pathogenesis of a variety of aging-related diseases. The cellular responses to oxidative stress, in particular those in response to sublethal doses of ROS, has not been thoroughly characterized. Several recent studies using human diploid fibroblasts showed that \(H_2O_2\) induced a permanent \(G_2\) growth arrest, which is phenotypically similar to replicative senescence (25–27). In this study, we investigated the nature of the transient cell cycle arrest induced by \(H_2O_2\) as a source of ROS, in mouse fibroblasts and go on to explore the mechanisms by which \(H_2O_2\) induced this proliferation arrest.

**EXPERIMENTAL PROCEDURES**

**Fibroblasts, MEF Isolation, and Cell Cultures**—Wild type mice and mice with deletion of p21\(^{Cip1}\) (28) were maintained at the animal facilities of the Imperial College (London, United Kingdom). Primary MEFs were isolated from 13.5 d embryos derived from the corresponding colonies of wild type or gene knock-out mice as described previously (29). Each embryo was dispersed and trypsinized for 20 min at 37 °C, and the resulting cells were grown for 1 day in a 10-cm-diameter tissue culture plate. After which, the cells were replated onto a 15-cm dish and allowed to grow for 2 days. These cells, designated passage number 0 cells, were stored in liquid nitrogen for later use. The MEFs were cultured and passaged as described previously (30). 10\(^6\) cells were replated every 3 days onto 100-mm plates. NIH 3T3, Swiss 3T3, and p21\(^{Cip1}\)-null (31) fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin. The NIH 3T3-D1, NIH 3T3-E, and NIH 3T3-K cell lines have been described previously (32), and exogenous cyclin expression was induced by incubation with 3- 

\[\text{IPTG} \quad (22)\]

for 24 h. \(H_2O_2\) was purchased from Sigma (H1009, 30% w/w solution) and was administered to the cells as a 10× solution in growth medium. For \(H_2O_2\) treatment, fibroblasts were grown to 60% confluenCe, and the tissue culture medium was changed before the addition of \(H_2O_2\).

**Cell Synchronization**—For synchronizing cells at the G1/G\(_2\) phase by serum deprivation (33), SWISS 3T3 fibroblasts were incubated in Dulbecco’s modified Eagle’s medium with 0.5% fetal calf serum for 48 h, before 10% fetal calf serum was added to induce the cells to re-enter the cell cycle. For synchronization at the G1/S transition, NIH 3T3 cells were first incubated with thymidine (25 mM; Sigma) for 16 h, released into the cell cycle for 12 h after removal of thymidine, and then treated again with thymidine for another 16 h. After the second block, thymidine was washed away and replaced with culture medium. Typically, 65–75% of cells re-entered the cell cycle and progressed into the S phase after the double thymidine block. To block cells at the metaphase/anaphase of M, the cells were treated with nocodazole (50 mg/ml; Sigma) for 16 h. The rounded up cells were detached by “mechanical shake-off,” washed, and resuspended in nocodazole-free culture medium for them to re-enter the cell cycle.

**Cell Cycle Analysis**—Cell cycle analysis was performed by combined propidium iodide and bromodeoxyuridine (BrdUrd) staining. Subconfluent fibroblasts with or without \(H_2O_2\) treatment were incubated for 30 min with 10 μM BrdUrd (Sigma). The cells were trypsinized, collected by centrifugation, and resuspended in PBS before fixing in 90% ethanol. The fixed cells were incubated first with 2 N HCl, then with 0.5 N Triton X-100 for 30 min at room temperature, and then with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibodies (BD Biosciences) at 1:3 dilution for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μg/ml propidium iodide (Sigma), 0.1 mg/ml RNase A (Sigma), 0.1% Nonidet P-40, and 0.1% Triton X-100 for 30 min at room temperature, and then with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibodies (BD Biosciences) at 1:3 dilution for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μg/ml propidium iodide (Sigma), 0.1 mg/ml RNase A (Sigma), 0.1% Nonidet P-40, and 0.1% Triton X-100 for 30 min at room temperature, and then with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibodies (BD Biosciences) at 1:3 dilution for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μg/ml propidium iodide (Sigma), 0.1 mg/ml RNase A (Sigma), 0.1% Nonidet P-40, and 0.1% Triton X-100 for 30 min at room temperature, and then with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibodies (BD Biosciences) at 1:3 dilution for 30 min, with PBS washes between each treatment. The cell cycle profile was analyzed using the Cell Quest software.

**Western Blot Analysis and Antibodies**—Western blot cell extracts were prepared by lysing cells with three times packed cell volume of lysis buffer (20 mM Hepes, pH 7.9, 150 mM NaCl, 1 mM MgCl\(_2\), 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM sodium orthovanadate) on ice for 20 min. The protein yield was quantified by Bio-Rad Dc protein assay kit (Bio-Rad). The samples corresponding to 50 μg of lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and recognized by appropriate antibodies. The antibodies against p21\(^{Cip1}\) (M-19), p27\(^{Kip1}\) (C-19), CDK4 (C-22), CDK6 (C-21), CDK2 (C-19), CDC2 (C-22), cyclin D1 (H-19), cyclin D3– (H-285), cyclin A (C-19), cyclin E (M-20), and cyclin B1 (M-143) were purchased from Santa Cruz Biotechnology. Anti-p27\(^{Kip1}\) (K25020) and anti-p21\(^{Cip1}\) (F-5) monoclonal antibodies were acquired from Transduction Laboratories and from Santa Cruz Biotechnology, respectively. The exogenous cyclins in the inducible NIH 3T3-D1, NIH 3T3-E, and NIH 3T3-K cell lines were detected by antibodies against FLAG (M2 from Sigma), human cyclin E (HE12 from Santa Cruz), and hemagglutinin (12CA5)
from Roche Molecular Biochemicals), respectively. Cdc1 was FLAG-tagged, the K cdc1 was hemagglutinin-tagged, and the human cdc1 can be distinguished from the endogenous mouse protein by the use of an antibody specific for human cdc1. The anti-α-tubulin, monoclonal TAT-1 has been described previously (32). The primary antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualized by the ECL detection system (Amersham Biosciences).

**Kinase Assays, Immunoprecipitation, and Immunodepletion—** For kinase assays, cells collected were washed with PBS and lysed in lysis buffer containing 50 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% Triton X-100, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 27 μg/ml leupeptin, 20 mM dithiothreitole, 10 mM NaF, 2 mM NaVO4, 200 μg of protein lysate was immunoprecipitated with 1-2 μg of antibody (CDC2, CDK4, cdc1, or cdk1 for CDK2 immunoprecipitation, 100 μg of protein, and 1 μg of antibody was used) and protein-Sepharose G beads (Amersham Biosciences) (50/ν v/v) overnight at 4 °C. The G beads were washed twice with lysis buffer and once with assay buffer (50 mM Hepes/NaOH, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM CAMP-dependent protein kinase inhibitor). The kinase assay was performed with 500 ng of histone H1/assay or C-terminal GST-Rb (C-terminal 792–928) (34) as substrate, 20 μM ATP, and 0.1 μCi of [γ-32P]ATP (3000 Ci/mmol; Amersham Biosciences) in 20 μl of assay buffer. The samples were then incubated for 30 min at 37 °C. The reaction was terminated with the addition of 50 μl of sample buffer and boiling for 3 min. The samples were resolved on a 12% gel (15 μl/15-well mini gel). The gel was then fixed and Comassie-stained, dried, and exposed to a PhosphorImager screen. Immunoprecipitation was performed as described for the kinase assay. For immunodepletion experiments, two extra immunoprecipitations were performed with either the anti-p21Cip1 or the anti-p27Kip1 monoclonal antibody cross-linked to protein G beads with the dimethylpimelidate method (35). The subsequent immunodepleted supernatants were then analyzed by Western blotting.

**Immunofluorescence and Mitotic Index Assay—** Cells cultured on coverslips were treated with or without 250 μM H2O2 for 4 h. The cells were washed with PBS, fixed with 4% formaldehyde, and then washed and permeabilized for 5 min with 0.5% Triton/10 mM dithiothreitole. The cells were then incubated for 1 h to overnight with monoclonal antibodies against p21Cip1 or p27Kip1, washed, and incubated with goat anti-mouse secondary FITC-conjugated antibodies (Molecular Bioprobes) to visualize the staining.

For mitotic index assay, exponentially growing fibroblasts were cultured on coverslips and synchronized by double thymidine block and release. The G1/M phase of these cells was trapped by addition of 100 ng/ml nocodazole (to trap cells that had progressed through G2 into mitosis) in the presence or absence of 250 μM H2O2. The cells were then stained and as above except 20 μg/ml of 4',6-diamidino-2-phenylindole (DAPI, Sigma) was added with the secondary antibody for 1 h to visualize the DNA. The nuclear morphology of the cells was analyzed by fluorescence microscopy. The number of cells displaying condensed chromosome morphology was counted and scored as mitotic and expressed as a percentage of the total.

**Northern Blot Analysis—** Total RNA was isolated using the RNeasy Kit (Qiagen) and quantified at 260 nm. 20 μg of RNA, prepared as above, was resolved on 1.5% formaldehydeagarose gels. Following electrophoresis, RNA were transferred to Hybond-N membrane (Amersham Biosciences) and subjected to Northern blotting as previously described (33). p21Cip1 mRNA was detected using hybridization with its full-length 32P-labeled mouse cDNA probe (36) kindly provided by Dr. B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). Actin mRNA was detected by a β-actin probe (37).

**Transfections and Gene Reporter Assays—** Transfection of NIH3T3 cells were performed using the calcium phosphate co-precipitation method as described previously (33). Briefly, calcium phosphate precipitates containing 10 μg of the wild type mouse p21Cip1 promoter-luciferase reporter plasmid (pGL3b-4542) (38) together with 2 μg of a β-galactosidase transfection control plasmid (pJ410/β-gal) (33) were incubated overnight with cycling 5 x 105 NIH3T3 cells in a 10-cm dish. The transfected NIH3T3 were then washed, treated with H2O2, and harvested for luciferase and β-galactosidase assays, as described previously (33).

**RESULTS**

**Sublethal Doses of H2O2 Induce a Transient Cell Cycle Arrest in NIH 3T3 Fibroblasts—** Previous studies showed that sublethal doses of H2O2 induced senescence-like permanent G1 cell cycle arrest in human fibroblasts 48 h after stimulation (26, 27). To investigate the more imminent and short term effects of H2O2 on cell cycle progression, we treated NIH 3T3 fibroblasts with sublethal doses (100–500 μM) of H2O2 and discovered that H2O2 caused cells to transiently arrest progression through the cell cycle (data not shown). Initial examination of the predominant staining alone indicated that there was no significant change in cell cycle distribution after H2O2 treatment. However, more detailed analysis of BrdUrd incorporation demonstrated a dramatic decrease in DNA synthesis detectable as early as 2 h after the addition of 250 μM of H2O2 (Fig. 1). DNA synthesis was almost completely abolished between 4–8 h after H2O2 treatment, after which the cells were temporarily delayed in the G2/M phases. At 48 h, the cells attained a more normal cell cycle profile, although proportionally more cells were found in the G1 phase, perhaps reflecting the build up of senescent-like cells previously reported (26). Notably, there was no apparent change in the proportion of cells in the G1, S, and G2/M phases of the cell cycle accompanying the transient arrest in DNA synthesis induced by the H2O2 treatment. This indicated that the H2O2-induced growth arrest was not confined only to the G1 phase and also involved other phases of the cell cycle. Taken together, these findings suggested that sublethal doses of H2O2 induced a rapid but transient multi-phase cell cycle arrest in mouse fibroblasts. To demonstrate that this effect was not restricted to the NIH3T3 cell line, we also examined the effect of H2O2 on the proliferation of low passage (<2 passages) MEFs and found that H2O2 also induced temporary cell cycle arrest in normal primary cells (see Fig. 8).

**H2O2 Triggers a Cell Cycle Arrest at G1, S, and Early G2/M Phases in SWISS 3T3 Fibroblasts Released from a G0 Block—** To confirm this hypothesis and investigate the nature of this multi-phase cell cycle arrest, we tested the ability of H2O2 to arrest fibroblasts at different phases of the cell cycle. To synchronize cells at different cell cycle phases, Swiss 3T3 fibroblasts were first arrested at G0 by serum starvation and then stimulated to re-enter the cell cycle by reinitiation of serum (Fig. 2). After serum stimulation, the fibroblasts transversed the late G1, early S, and G2/M phases of the cell cycle at 12, 16, and 24 h, respectively. At 28 h after serum stimulation, the majority of the cells re-entered the G1 phase from the G2/M
phase (Fig. 2A). To study the effects of \( \text{H}_2\text{O}_2 \) on different cell cycle phases, these serum-stimulated cells were either untreated or treated with 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) and harvested 4 h later for cell cycle analysis. \( \text{H}_2\text{O}_2 \) prevented the fibroblasts from progressing further through the cell cycle when added to cells at the G1 and S phases (12, 16, and 20 h post-serum stimulation) (Fig. 2A). The results showed that when \( \text{H}_2\text{O}_2 \) was added to the serum-stimulated cells at 24 h, a substantial number of the cells continued to progress into G1 from G2/M (Fig. 2A). This indicated that most G2/M cells were insensitive to \( \text{H}_2\text{O}_2 \)-induced cell cycle arrest. In a second independent experiment (Fig. 2B), the number of cells entering G1 following treatment with \( \text{H}_2\text{O}_2 \) at 24 h post-serum stimulation was significantly smaller than their untreated counterparts. This is likely to reflect the fact that the majority of the cells used in Fig. 1 were slightly further advanced in the cell cycle than those in Fig. 2B and were in the M phase rather than the G2 phase (see below).

Because some of the cells in the G2/M phase did progress into the G1 phase as illustrated in Fig. 2A, this cell cycle block was unlikely to be late in the M phase. These observations indicated that \( \text{H}_2\text{O}_2 \) induced growth arrest in cells at the G1, S, and early G2/M phases.

\( \text{H}_2\text{O}_2 \) Triggers NIH 3T3 Cells to Arrest at G1, S, and Early G2/M, but Not M Phase—Next we tested the effects of \( \text{H}_2\text{O}_2 \) on NIH 3T3 fibroblasts released from a nocodazole-induced M phase block (Fig. 3). The results showed that pretreatment with 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) failed to prevent the fibroblasts from progressing from M to G1 phase because both the untreated and \( \text{H}_2\text{O}_2 \)-treated cells escaped into G1 at similar kinetics after released from M. It was also found that \( \text{H}_2\text{O}_2 \) only became effective in imposing growth arrest once the cells progressed from the M phase into the G1 and S phases of the cell cycle. Consistent with earlier results, these results confirmed that sublethal doses of \( \text{H}_2\text{O}_2 \) induced a rapid but transient growth arrest at the G1, S, and early G2/M phases.

\( \text{H}_2\text{O}_2 \) Blocks Cells at G2 but Not M Phase—To determine precisely where in the G2/M phase \( \text{H}_2\text{O}_2 \) induces this transient cell cycle arrest, we examined whether \( \text{H}_2\text{O}_2 \) treatment could block cells from progressing from the G2/M into M phase. To achieve this, we first arrested NIH 3T3 cells at the G/S boundary using a double thymidine cell cycle block and then released them to progress through the cell cycle (Fig. 4). The majority of these synchronized cells acquired a 4N DNA content and thus reached G2/M at 6 h after release from the G/S block. It was notable that a proportion of the cells did not leave G/S after release from the second thymidine block. However, these cells never re-entered the cell cycle and consequently did not interfere with the G2/M synchronized population of cells. The cells traversing G2/M at 6 h after release from G/S were subjected to nocodazole treatment in the presence or absence of 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). Nocodazole (50 ng/ml) was added to trap cells at metaphase to allow the analysis of the percentage of cells that had progressed from G2 into mitosis. At 4 h after the addition of nocodazole in the presence or absence of \( \text{H}_2\text{O}_2 \), the cells were fixed, and the percentage of mitotic cells was determined following immunofluorescence staining using the DNA-intercalating fluorochrome DAPI (Fig. 4B). Propidium iodide staining showed that the proportion of cells arrested at G2/M (with 4N DNA content) is similar with or without \( \text{H}_2\text{O}_2 \) treatment (Fig. 4). It was also found that the percentage of mitotic cells

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**FIG. 2.** Cell cycle analysis of Swiss 3T3 fibroblasts synchronized by serum deprivation after \( \text{H}_2\text{O}_2 \) treatment. Swiss 3T3 fibroblasts arrested at G1 by serum deprivation were stimulated to re-enter the cell cycle with 10% fetal calf serum. The cells were labeled with BrdUrd, fixed, stained with propidium iodide and FITC-conjugated anti-BrdUrd antibodies, and processed for FACS analysis as in Fig. 1. The upper panel shows the cell cycle profile of fibroblasts at 0, 12, 16, 20, 24, and 28 h after serum stimulation. Swiss 3T3 fibroblasts at 12, 16, 20, 24, and 28 h after serum stimulation were treated with 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) and harvested 4 h later for cell cycle analysis. The lower panel reveals the cell cycle status of cells 4 h later for cell cycle analysis. H2O2 prevented the fibroblasts from progressing from M into G1 phase because both the untreated and \( \text{H}_2\text{O}_2 \)-treated cells were harvested after 4 h, the cells at 16, 20, 24, and 28 h after serum stimulation served as the untreated control of the cells treated with \( \text{H}_2\text{O}_2 \) at 12, 16, 20, and 24 h, respectively. B, a duplicated experiment as above. However, only the cell cycle profile of fibroblasts 24 h after serum stimulation and 4 h afterward (28 h after serum stimulation) with or without \( \text{H}_2\text{O}_2 \) treatment is shown.
H$_2$O$_2$ Induces Multi-phase Transient Cell Cycle Arrest

We next proceeded to investigate the mechanism involved in this multi-phase cell cycle arrest. To achieve this, we analyzed the expression of different components of the cyclin-dependent kinase complexes, which are important for regulating cell cycle progression, following treatment with 250 $\mu$M H$_2$O$_2$ (Fig. 5). The Western blot results showed that although H$_2$O$_2$ increased cyclin D1 and D3 expression, following H$_2$O$_2$ treatment, cyclin D1 and D3 expression was significantly lower in the H$_2$O$_2$-treated fibroblasts than their untreated counterparts (Fig. 4B), even though the total number of cells arrested at G2/M with and without H$_2$O$_2$ were comparable, as revealed by the propidium iodide staining (Fig. 4A). The results indicated that almost all H$_2$O$_2$-treated cells with 4 N DNA content resulted from a cell cycle block at G2 but not M. This finding therefore suggests that H$_2$O$_2$ inhibits cell proliferation at the G2 but not the M phase of the cell cycle.

The H$_2$O$_2$-induced Cell Cycle Arrest Is Associated with Down-regulation of Cyclin Ds and Up-regulation of p21Cip1 Expression—We next proceeded to investigate the mechanism involved in this multi-phase cell cycle arrest. To achieve this, we analyzed the expression of different components of the cyclin-dependent kinase complexes, which are important for regulating cell cycle progression, following treatment with 250 $\mu$M H$_2$O$_2$ (Fig. 5). The Western blot results showed that although there was no obvious change in the expression levels of cyclins E and A, CDK2, CDK4, and CDC2 in response to H$_2$O$_2$ stimulation, there was a significant reduction in cyclin D1 and D3 expression. Following H$_2$O$_2$ treatment, cyclin D1 and D3 expression reached a nadir at 4 h, before recovering to higher levels. Moreover, it was found that the CKI p21Cip1 was up-regulated by H$_2$O$_2$ treatment and that its expression peaked at 4 h after H$_2$O$_2$ treatment. Notably, cyclin D2 and p57Kip2 expression is not detectable in these mouse fibroblasts (data not shown). Interestingly, the kinetics for down-regulation of cyclin D1 and D3 and up-regulation of p21Cip1 coincided with that of the H$_2$O$_2$-induced growth arrest, indicating that the cyclin D1 and D3 and p21Cip1 could have a role in mediating this transient cell cycle arrest. It was notable that the expression level of another CKI, p27Kip1, decreased after H$_2$O$_2$ stimulation and was inversely correlated with cell cycle arrest, suggesting that p27Kip1 is therefore unlikely to be involved in the cell cycle arrest induced by H$_2$O$_2$.

The Induction of p21Cip1 by H$_2$O$_2$ Occurs at Transcriptional Level but Does Not Require p53 Function—To investigate the mechanism for p21Cip1 regulation by H$_2$O$_2$, we performed Northern blot analysis on NIH3T3 treated with H$_2$O$_2$ (Fig. 5). The results showed that H$_2$O$_2$ increased p21Cip1 mRNA levels with kinetics similar to that of the p21Cip1 protein, indicating that p21 transcription increases is a component of the H$_2$O$_2$ response. To test whether this induction of p21Cip1 expression by H$_2$O$_2$ was also mediated at gene promoter level, we transiently transfected NIH 3T3 cells with a p21Cip1 promoter/luciferase reporter construct and monitored the luciferase activity following treatment with 250 $\mu$M H$_2$O$_2$ (Fig. 5). p21Cip1 promoter activity essentially paralleled the changes in p21Cip1 mRNA levels, indicating that the induction of p21Cip1 in response to H$_2$O$_2$ treatment can be largely accounted for through alterations in transcription rate, although other mechanisms have not been eliminated.

The CKI p21Cip1 is one of the primary transcriptional targets of the tumor suppressor p53. p53 has previously been shown to mediate G1 arrest induced by DNA damage through activating p21Cip1 gene transcription, and it is possible that the induction of p21Cip1 expression by H$_2$O$_2$ was mediated through p53. Indeed, immunoblot analysis (Fig. 5) indicated that p53 levels did increase in response to H$_2$O$_2$ treatment. However, p53 expres-
Progression through the cell cycle—

**A**

![Diagram A](http://www.jbc.org/content/1103/2/13766/F5.large.jpg)

**B**

![Diagram B](http://www.jbc.org/content/1103/2/13766/F6.large.jpg)

**C**

![Diagram C](http://www.jbc.org/content/1103/2/13766/F6.large.jpg)

**Fig. 5.** Expression of cell cycle regulators in NIH 3T3 fibroblasts following H₂O₂ treatment. A, cell lysates were prepared from NIH 3T3 fibroblasts at the times indicated following treatment with 250 μM H₂O₂. The expression of cyclin D1, D3, E, A, and B, CDK2, CDK4, CDC2, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, p53 were analyzed by Western blotting. Total RNA was isolated in parallel, and the expression of p21<sup>Cip1</sup> and actin mRNA was determined by Northern blotting. B, a wild type p21<sup>Cip1</sup> promoter-luciferase construct was transfected into cycling NIH 3T3 cells. The transfected cells were treated with 250 μM H₂O₂ collected 4 h later, and lysed for luciferase assay. The luciferase activity was normalized with β-galactosidase activity specified by co-transfected p4thβ-gal plasmid and expressed as the mean ± S.D. of at least three independent experiments each with duplicated transfection. C, lysates were prepared from p53<sup>−/−</sup> MEFs at the times indicated following treatment with 250 μM H₂O₂, and the expression of cyclin D1 and D3, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> was analyzed by Western blotting. Expression subsided before the accumulation of p21<sup>Cip1</sup> at either protein or mRNA levels, indicating that the induction of p21<sup>Cip1</sup> expression was not a direct consequence of p53 accumulation. To further confirm this idea, we treated MEFs derived from p53<sup>−/−</sup> mice with 250 μM H₂O₂ and followed the expression patterns of p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and cyclin Ds. Western blot results showed that the induction of p21<sup>Cip1</sup> expression by H₂O₂ in p53<sup>−/−</sup> MEFs occurred at similar kinetics as in the NIH 3T3 fibroblasts, indicating that p53 is not essential for the induction of p21<sup>Cip1</sup> expression by H₂O₂. Likewise, the down-regulation of p27<sup>Kip1</sup> and cyclin D1 and D3 expression also occurred in the absence of p53 following H₂O₂ stimulation.

**The H₂O₂-induced Cell Cycle Arrest Is Associated with Repression of Cyclin D-CDK4/6, Cyclin E-CDK2, Cyclin A-CDK2, and Cyclin B-CDC2 Activity—**Progression through the cell cycle from G<sub>1</sub> to M requires the sequential activation of cyclin D-CDK4/6, cyclin E-CDK2, cyclin A-CDK2, cyclin B-CDC2 activity, which are important for transition through G<sub>1</sub>, initiation of S, advance through S, and passage from G<sub>2</sub> to M, respectively (12, 38–42). Our data showed that H₂O₂ induced multi-phase cell cycle arrest at G<sub>1</sub>, S, and G<sub>2</sub>M. Consequently, we next examined the effects of H₂O₂ on the kinase activity of the cyclin D-CDK4, cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDC2 complexes. CDK complexes were immunoprecipitated using specific anti-cyclin and CDK antibodies and the levels of cyclin and CDK-associated kinase activity measured against a bacterially synthesized pRB fragment (Rb 792–973) or histone H1 as substrates (Fig. 6A). The immunoprecipitation kinase assays showed that although the activity of cyclins A, E, and B, CDK4, CDK2, and CDC2 containing kinase complexes remained at high levels in untreated fibroblasts, these cyclin-CDK complexes were significantly inactivated in the H₂O₂-treated cells (Fig. 6A). Collectively, the kinase assays showed that the H₂O₂-induced multi-phase growth arrest was correlated with down-regulation of cyclin D-CDK4-, cyclin A- and E-CDK2-, and cyclin B-CDC2-associated kinase activity. Given that the level of CDK4 expression remained unchanged before and after H₂O₂ stimulation, the decrease in cyclin D-CDK4 activity is likely to be primarily the result of the down-regulation of cyclin D1, cyclin D2, and cyclin D3 expression induced by H₂O₂, although the involvement of other mechanisms could not be excluded.

The WAF/KIP family of CKIs, including p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, have been shown to trigger cell cycle arrest through specifically binding to and inhibiting CDK2 kinase complexes. Therefore, the reduction of cyclin A and E-CDK2 activity could be due to the induction of p21<sup>Cip1</sup> by H₂O₂. To explore further the roles of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in the H₂O₂-induced cell cycle arrest, we used anti-p21<sup>Cip1</sup> or anti-p27<sup>Kip1</sup> antibodies cross-linked to Sepharose G beads to immunodeplete cyclin-dependent kinase complexes from NIH 3T3 fibroblasts before and 4 h after H₂O₂ treatment. The subsequent immunodepleted lysates were then Western blotted for components of cyclin associated kinase
complexes (Fig. 6B). The result showed that significant levels of cyclins A and E and CDK2 remained in the untreated cell lysates after p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) immunodepletion, indicating that in untreated cells the majority of the cyclin E-CDK2 and cyclin A-CDK2 complexes are “free” of these two CKIs. It also demonstrated that although the anti-p21\(^{\text{Cip1}}\) antibodies failed to deplete any of the cyclin A/E-CDK2 complexes in the untreated cells, they removed the majority of the cyclins A and E and CDK2 proteins from the H\(_2\)O\(_2\)-treated cell lysates. This indicates that the majority of the cyclin A- or E-CDK2 complexes are free in the untreated cells but are associated with p21\(^{\text{Cip1}}\) following H\(_2\)O\(_2\) stimulation. In contrast, although the anti-p27\(^{\text{Kip1}}\) antibodies effectively removed all p27\(^{\text{Kip1}}\) protein from the untreated and H\(_2\)O\(_2\)-treated cell lysates, the antibodies failed to eliminate the cyclins and dependent kinases, including cyclins Ds, A, E, and B, CDK2, CDK4, and CDC2, before and after H\(_2\)O\(_2\) treatment. These results not only indicated that H\(_2\)O\(_2\) treatment increased the amount of cyclin A- or E-CDK2 complexes associated with p21\(^{\text{Cip1}}\) but also suggested that the majority of the cyclin A- or E-CDK2 complexes were bound to p21\(^{\text{Cip1}}\) after H\(_2\)O\(_2\) stimulation. It was also found that the anti-p27\(^{\text{Kip1}}\) antibodies depleted cyclin D1 and CDK4 in cell lysates with and without H\(_2\)O\(_2\) treatment. Conversely, little or no cyclin B and CDC2 was eliminated by the anti-p21\(^{\text{Cip1}}\) antibodies with or without H\(_2\)O\(_2\) treatment. Although our result showed that there was no binding of p21 to the cyclin B-CDC2 complex, previous studies have shown that increased levels of p21 can induce a G2 arrest by inhibiting the CAK-mediated Thr\(^{161}\) phosphorylation of CDC2. Thus, the G2 arrest could also be attributable to the induction of p21 expression by H\(_2\)O\(_2\).

\(p21^{\text{Cip1}}\) Localized Exclusively in the Nucleus and \(p27^{\text{Kip1}}\) Largely in the Cytoplasm—Interestingly, although p27\(^{\text{Kip1}}\) was present abundantly in the cells both before and after H\(_2\)O\(_2\) treatment, they failed to associate with the cyclin-CDK complexes. The subcellular localization of p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) plays a part in regulating their activity, and it is possible that the absence of p27\(^{\text{Kip1}}\) binding to cyclin A and E-CDK2 could be due to the fact that p27\(^{\text{Kip1}}\) and the cyclin-dependent kinases are not present in the same subcellular compartments. Consistent with this, it has been documented previously that p27\(^{\text{Kip1}}\) is localized in the cytoplasm of proliferating Swiss 3T3 cells (43). To investigate this possibility, we studied the subcellular localization of p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) before and 4 h after H\(_2\)O\(_2\) treatment by immunofluorescence staining using antibodies specific for p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) (Fig. 7). Immunofluorescence experiments showed that p21\(^{\text{Cip1}}\) localized primarily within the nucleus and p27\(^{\text{Kip1}}\) in the cytoplasm and that H\(_2\)O\(_2\) treatment did not affect the subcellular localization of either p21\(^{\text{Cip1}}\) or p27\(^{\text{Kip1}}\) (Fig. 7). In accordance with the Western blotting results, p21\(^{\text{Cip1}}\) expression was higher in the H\(_2\)O\(_2\)-treated cells compared with the untreated cells, where p21\(^{\text{Cip1}}\) levels were very low. Because the cyclin-CDK complexes are located predominantly in the nucleus, the cytoplasmic localization of p27\(^{\text{Kip1}}\) could help explain the lack of binding of p27\(^{\text{Kip1}}\) to the cyclin A/E-CDK2 complexes. Studies are currently underway to address the significance of this observation.

Ectopic Expression of K Cyclin, but Not Cyclin D1 or Cyclin E, Can Rescue Fibroblasts from the H\(_2\)O\(_2\)-induced Cell Cycle Block—On the basis of our findings, we propose a model in which moderate levels of H\(_2\)O\(_2\) induce a transient multi-phase cell cycle arrest through up-regulation of p21\(^{\text{Cip1}}\) and down-regulation of cyclin D expression. To investigate the contribution of p21\(^{\text{Cip1}}\) in this H\(_2\)O\(_2\)-induced cell cycle arrest, MEFs from wild type and p2\(^{\text{G1}}\) mice were treated with 250 \(\mu\)M H\(_2\)O\(_2\) (Fig. 8). The results showed that after H\(_2\)O\(_2\) treatment, normal MEFs and MEFs lacking p21\(^{\text{Cip1}}\) underwent cell cycle arrest with similar kinetics. This result indicated that deletion of p21\(^{\text{Cip1}}\) function alone is not sufficient to overcome the cell cycle arrest mediated by H\(_2\)O\(_2\) and highlighted the fact that the H\(_2\)O\(_2\)-induced cell cycle arrest also involved down-regulation of cyclin D, cyclin E, or a viral K cyclin is enough to overcome the cell cycle arrest imposed by low concentrations of H\(_2\)O\(_2\). Overexpression of cyclin D, cyclin E, or a viral K cyclin is enough to overcome the cell cycle arrest induced by H\(_2\)O\(_2\). K cyclin is a human herpesvirus 8 viral cyclin that can mimic the function of cyclin Ds and is resistant to CKIs, including p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\). To this end, we used NIH 3T3 fibroblast cell lines that expressed cyclin D1, cyclin E, or K cyclin, upon the addition of IPTG (32). The inducible expression of cyclin D1, cyclin E, and K cyclin in these three NIH 3T3 fibroblast cell lines by IPTG stimulation was demonstrated by Western blot analysis of serum-deprived quiescent cells in the presence of IPTG stimulation (Fig. 9). The cells were incubated in the absence or presence of 250 or 500 \(\mu\)M H\(_2\)O\(_2\) for 4 h, with and without prior induction of ectopic cyclin expression. After treatment, the cells were harvested for cell cycle analysis (Fig. 10). Cyclin E was unable to effectively override the cell cycle arrest induced by H\(_2\)O\(_2\). Overexpression of cyclin D1 appeared to be able to partially revert the cell cycle arrest imposed by low concentrations of H\(_2\)O\(_2\) (250 \(\mu\)M), as revealed by the low levels of BrdUrd incorporation in the presence cyclin D1 expression following H\(_2\)O\(_2\) treatment. This ability of cyclin D1 to partially
is a cessation of DNA synthesis. This cell cycle arrest was apparent 2 h after exposure to H$_2$O$_2$ and lasted for about 6 h.

To characterize further the nature of this cell cycle arrest, we examined the effects of H$_2$O$_2$ on fibroblasts optimally synchronized at different phases of the cell cycle using three different methods: serum deprivation, nocodazole, and thymidine blocks. Our results suggested that H$_2$O$_2$ activates cell cycle checkpoints at G$_1$ and S phases and somewhere within the G$_2$/M phases of the cell cycle. The observation that some, although not all, G$_2$/M cells entered G$_2$ after H$_2$O$_2$ stimulation suggests that H$_2$O$_2$ arrests cells somewhere in the early part of G$_2$/M. Consistent with this are the subsequent findings that H$_2$O$_2$ failed to delay cells released from a nocodazole induced M phase block but prevented cells entering M from G$_2$, thus confirming that H$_2$O$_2$ arrests cells at the G$_2$ but not at the M phase. Together, these synchronized cell cycle phase experiments show that H$_2$O$_2$ activates cell cycle checkpoints in the G$_1$, S, and G$_2$ phases but not in the M phase of the cell cycle.

The molecular mechanisms involved in the H$_2$O$_2$ response appear complex. Our initial experiments indicated the involvement of the D-type cyclins and the CKI p21Cip1. D-type cyclin expression was marked down-regulated in response to H$_2$O$_2$ and resulted in a reduction in cyclin D-CDK4 activity. Because the progression of cells from G$_1$ to S is initiated by expression of D-type cyclins and their assembly with CDK4, the down-regulation of cyclin D1 and D3 expression and their associated CDK4 activity by H$_2$O$_2$ can explain the G$_1$ phase arrest induced by H$_2$O$_2$. Likewise, the down-regulation of cyclin E-CDK2 and cyclin A-CDK2 activity by H$_2$O$_2$ could account for the block of DNA synthesis caused by H$_2$O$_2$. Interestingly, the down-regulation of cyclin E-CDK2 and cyclin A-CDK2 activity by H$_2$O$_2$ is not accompanied by significant changes in cyclin A and E and CDK2 levels. This reduction in cyclin E/A-CDK2 after H$_2$O$_2$ is predominantly a result of increase in p21Cip1 binding to cyclin E/A-CDK2. The increase in binding of p21Cip1 to cyclin E/A-CDK2 is primarily the result of increased transcription of the p21Cip1 gene following H$_2$O$_2$ treatment. p21Cip1 expression can be activated by p53-dependent and independent mechanisms in response to cellular stresses, such as DNA damage, to mediate G$_1$ cell cycle arrest (45–47). In the present study, we showed that H$_2$O$_2$ can induce transient cell cycle arrest and up-regulation of p21Cip1 in the absence of p53 function. Nevertheless, we still cannot exclude the possibility that p53 contributes to the H$_2$O$_2$-induced p21Cip1 expression and cell cycle arrest in normal cells.

Cell cycle progression from G$_2$ to M phase is regulated by cyclin A/B-CDK2 activity (14, 42, 48); therefore, the G$_2$ arrest following H$_2$O$_2$ treatment can be attributable to the decrease in cyclin A/B-CDK2 associated kinase activity observed after H$_2$O$_2$ treatment. However, this decrease in cyclin A/B-CDK2 activity is not accompanied by any detectable change in cyclin A and BCDK2 expression. Previous studies have shown that increased levels of p21Cip1 can induce a G$_2$ arrest by inhibiting the CAK (CDK-activating kinase)-mediated Thr$^{161}$ phosphorylation of CDC2 (49). As a result, the H$_2$O$_2$-induced accumulation of p21Cip1 could also be at least in part responsible for down-regulation of cyclin A/B-CDK2-dependent kinase activity and the consequent G$_2$ arrest induced by H$_2$O$_2$. Consistent with our finding, this inhibitory function of p21Cip1 does not require its physical association with cyclin B or CDC2 (49). Indeed, over-expression of p21Cip1 has been demonstrated to be able to enforce a G$_2$ as well as G$_1$ cell cycle arrest through inhibiting the cyclin A-, E-, and B-associated kinase activity (49, 50). Dephosphorylation of two inhibitory residues, Thr$^{14}$ and Tyr$^{15}$, is required for the G$_2$ to M transition in mammalian cells, and the cyclin B-CDC2 complex is inactive when CDC2 is phospho-
gested that p21Cip1 and PCNA co-operate to maintain cell cycle 
progression. Our immunofluorescence staining reveals that p21 Cip1 locates predominantly in the nucleus, whereas p27Kip1 accumulates mainly in the cytoplasm regardless of H$_2$O$_2$ treatment. Collectively, these data showed that p21Cip1 expression contributes to the multi-phase cell cycle arrest induced by H$_2$O$_2$. To establish further the roles of p21Cip1 and D-type cyclins in this transient cell cycle arrest induced by H$_2$O$_2$ treatment, we tested whether deletion of p21Cip1 or ectopic expression of cyclin D1 or E can override the cell cycle arrest induced by H$_2$O$_2$. However, using p21Cip1 null MEFs and NIH 3T3 cells that express cyclin D1 or E under the control of an inducible promoter, we showed that deletion of p21Cip1, restoration of cyclin D expression, or overexpression of cyclin E alone is insufficient to effectively overcome the cell cycle arrest caused by sublethal doses of H$_2$O$_2$. By contrast, overexpression of a human herpesvirus 8 K cyclin is enough to override this transient cell cycle arrest. Although it has been shown that the cyclin K-CDK6 is resistant to p21Cip1 inhibition and can mimic cyclin E-CDK2 activity with respect to DNA synthesis initiation (32, 58), it is conceivable that K cyclin can also interfere with other cell cycle regulators to overcome this transient multi-phase cell cycle arrest. The observation that H$_2$O$_2$ can still cause S phase arrest in p21Cip1 mutant cells suggests that DNA damage-induced S phase arrest may be independent of p21Cip1. However, in p21Cip1 null MEFs and NIH 3T3-K cell lines, a significant level of p53 was detected by Western blot analysis prior and after IPTG stimulation, indicating that p53 is involved in the multi-phase cell cycle arrest caused by sublethal doses of H$_2$O$_2$. Despite this, p53 deletion does not completely rescue the cell cycle arrest in p21Cip1 null MEFs, suggesting that other mechanisms are also involved.

The down-regulation of D-type cyclin and up-regulation of p21Cip1 expression contributes to the multi-phase cell cycle arrest induced by H$_2$O$_2$. To establish further the roles of p21Cip1 and D-type cyclins in this transient cell cycle arrest induced by H$_2$O$_2$ treatment, we tested whether deletion of p21Cip1 or ectopic expression of cyclin D1 or E can override the cell cycle arrest induced by H$_2$O$_2$. However, using p21Cip1 null MEFs and NIH 3T3 cells that express cyclin D1 or E under the control of an inducible promoter, we showed that deletion of p21Cip1, restoration of cyclin D expression, or overexpression of cyclin E alone is insufficient to effectively overcome the cell cycle arrest caused by sublethal doses of H$_2$O$_2$. By contrast, overexpression of a human herpesvirus 8 K cyclin is enough to override this transient cell cycle arrest. Although it has been shown that the cyclin K-CDK6 is resistant to p21Cip1 inhibition and can mimic cyclin E-CDK2 activity with respect to DNA synthesis initiation (32, 58), it is conceivable that K cyclin can also interfere with other cell cycle regulators to overcome this transient multi-phase cell cycle arrest. The observation that H$_2$O$_2$ can still cause S phase arrest in p21Cip1 mutant cells suggests that DNA damage-induced S phase arrest may be independent of p21Cip1. However, in p21Cip1 null MEFs and NIH 3T3-K cell lines, a significant level of p53 was detected by Western blot analysis prior and after IPTG stimulation, indicating that p53 is involved in the multi-phase cell cycle arrest caused by sublethal doses of H$_2$O$_2$. Despite this, p53 deletion does not completely rescue the cell cycle arrest in p21Cip1 null MEFs, suggesting that other mechanisms are also involved.

**FIG. 10. Analysis of the ability of cyclins D1, E, and K to overcome the cell cycle arrest induced by H$_2$O$_2$ treatment.** NIH 3T3-D1, NIH 3T3-E, or NIH 3T3-K cell lines were either left untreated (-) or treated (+) with 3 mM IPTG for 24 h to induce cyclin expression. These IPTG-pretreated cells as well as the untreated cells were then treated with either 0, 250, or 500 $\mu$M H$_2$O$_2$ prior to collection for cell cycle analysis 4 h after induction. BrdUrd was added to the cells for the final 30 min of the experiment. The harvested cells were fixed and stained with propidium iodide and FITC-conjugated anti-BrdUrd antibodies (lower panels) prior to FACS analysis to measure DNA contents and to determine the cells in DNA synthesis, respectively. The percentages of cells in each cell cycle phase (G1, S, and G2/M) determined by DNA contents and stained positive for BrdUrd are indicated.
multi-phase cell cycle arrest induced by \( \text{H}_2\text{O}_2 \) exposure. Collectively, our results show that sublethal doses of \( \text{H}_2\text{O}_2 \) induce a rapid and transient growth arrest at the \( \text{G}_1 \), \( \text{S} \), and \( \text{G}_2 \) phases of cell cycle in mouse fibroblasts. Our data also demonstrated that this multi-phase cell cycle arrest is, at least in part, mediated by the down-regulation of D-type cyclin and induction of \( p21\text{Cip1} \) expression, culminating in the inhibition of CDK4, CDK2, and CDC2-associated kinase activities.

A previous study on human diploid fibroblasts showed that sublethal levels of \( \text{H}_2\text{O}_2 \) induce a long term senescence related cell cycle arrest at \( \text{G}_1 \) phase of the cell cycle (26). This report documents the long term more permanent responses of human fibroblasts to \( \text{H}_2\text{O}_2 \), whereas our present study concentrates on the more immediate and temporary effects of \( \text{H}_2\text{O}_2 \). Although some similarities are shared between the two studies, it is evident that many differences exist. Most noticeably, in these studies may reside wholly in the fact that the present study was performed on cells of human origin (26). Although the oxidative stress-induced transient cell cycle arrest is beginning to attract increasing attention in recent years, the nature of this cell cycle arrest and molecular mechanisms by which this growth arrest is mediated remained very much undefined. To our knowledge, the present study represents the first detailed investigation on the nature and molecular basis of the \( \text{H}_2\text{O}_2 \)-induced transient cell cycle arrest.

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REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922
2. Cerutti, P. A. (1994) Science 267, 375–381
3. Cerutti, P. A. (1993) J. Cell. Biochem. 48, 41–47
4. Davies, K. J. (1995) J. Biol. Chem. 270, 27824–27827
5. Finkel, T. (2000) FEBS Lett. 476, 52–54
6. Sherr, C. J., and Roberts, J. M. (1999) Mol. Cell. Biol. 10, 42–45
7. Sardet, C., LeCam, L. E., Fabbrizio, E., and Vidal, M. (1997) in Oncogenes as Transcriptional Regulators (Gyhdrad, Y., and Yaniv, M., eds) Vol. 2, pp. 1–63, Birkhauser Verslag, Berlin, Germany
8. Dyson, N. (1998) Genes Dev. 12, 2245–2262
9. Chen, M., Olivier, P., Diehl, J. A., Fero, M., Rousset, M. F., Roberts, J. M., and Sherr, C. J. (1999) EMBO J. 18, 1571–1583
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REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922
2. Cerutti, P. A. (1994) Science 267, 375–381
3. Cerutti, P. A. (1993) J. Cell. Biochem. 48, 41–47
4. Davies, K. J. (1995) J. Biol. Chem. 270, 27824–27827
5. Finkel, T. (2000) FEBS Lett. 476, 52–54
6. Sherr, C. J., and Roberts, J. M. (1999) Mol. Cell. Biol. 10, 42–45
7. Sardet, C., LeCam, L. E., Fabbrizio, E., and Vidal, M. (1997) in Oncogenes as Transcriptional Regulators (Gyhdeal, Y., and Yaniv, M., eds) Vol. 2, pp. 1–63, Birkhauser Verslag, Berlin, Germany
8. Dyson, N. (1998) Genes Dev. 12, 2245–2262
9. Chen, M., Olivier, P., Diehl, J. A., Fero, M., Rousset, M. F., Roberts, J. M., and Sherr, C. J. (1999) EMBO J. 18, 1571–1583
10. Vogelstein for the mouse \( p21 \text{Cip1} \) cDNAs, Dr. M. Serrano for the \( p21\text{Cip1} \) expression, culminating in the inhibition of CDK4, CDK2, and CDC2-associated kinase activities.

A previous study on human diploid fibroblasts showed that sublethal levels of \( \text{H}_2\text{O}_2 \) induce a long term senescence related cell cycle arrest at \( \text{G}_1 \) phase of the cell cycle (26). This report documents the long term more permanent responses of human fibroblasts to \( \text{H}_2\text{O}_2 \), whereas our present study concentrates on the more immediate and temporary effects of \( \text{H}_2\text{O}_2 \). Although some similarities are shared between the two studies, it is evident that many differences exist. Most noticeably, in these studies may reside wholly in the fact that the present study was performed on cells of human origin (26). Although the oxidative stress-induced transient cell cycle arrest is beginning to attract increasing attention in recent years, the nature of this cell cycle arrest and molecular mechanisms by which this growth arrest is mediated remained very much undefined. To our knowledge, the present study represents the first detailed investigation on the nature and molecular basis of the \( \text{H}_2\text{O}_2 \)-induced transient cell cycle arrest.

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H$_2$O$_2$ Induces a Transient Multi-phase Cell Cycle Arrest in Mouse Fibroblasts through Modulating Cyclin D and p21 Cip1 Expression

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