# Cyclin D1 degradation enhances endothelial cell survival upon oxidative stress

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## SPECIFIC AIMS

Enhanced oxidative damage to endothelial cells (EC) is a prominent feature of many physiopathological conditions. Indeed, reactive oxygen species (ROS) have been shown to mediate EC apoptosis in a variety of cardiovascular diseases, including ischemia/reperfusion injury, diabetic vasculopathy, hypertension, atherosclerosis, and heart failure.

Several proteins that stimulate cell cycle progression have been demonstrated to induce apoptosis when deregulated. Specifically, the *cyclin D1* gene (*CCND1/BCL1/PRAD1*) plays an integral part in cell growth and survival control. In this study, we investigated how oxidative stress regulates cyclin D1 turnover and the functional relevance of cyclin D1 degradation for EC survival.

## PRINCIPAL FINDINGS

### 1. D-cyclins are down-regulated in response to oxidative stress

Human umbelical vein EC (HUVEC) were treated with 400 μmol/l $H_2O_2$ for 1–24 h: after 2 h of $H_2O_2$ treatment, cyclin D1 accumulation decreased significantly (23±2% of control; $P<0.001$; **Fig. 1*A***) and low levels of this cyclin were maintained up to 8 h after treatment. Thereafter, cyclin D1 expression recovered, returning to control levels 24 h after $H_2O_2$ addition. Cyclin D3 protein was similarly modulated, albeit to a lower extent, while cyclin D2 was undetectable.

D-cyclin down-modulation was observed both at sublethal (200–400 μmol/l) and lethal (800 μmol/l) doses of $H_2O_2$ (Fig. 1*B*) and occurred with similar kinetics in other EC (bovine artery EC and porcine artery EC) and non-EC types (U2OS osteosarcoma cell line). Interestingly, up to 8 h of $H_2O_2$ treatment did not modulate protein levels of cyclins A, B, and E.

We also tested whether other interventions causing oxidative stress induced D-cyclins down-modulation.

Cell treatment with the alkylating chemotherapic drug 1,3-bis-(2 chloroethyl)-1-nitrosourea (BCNU) causes glutathione (GSH) reductase inhibition and the decrease of reduced-to-oxidized GSH ratio. HUVEC incubation with 0.25 mmol/l BCNU for 2 h caused cyclin D1 down-regulation, and this phenomenon was inhibited by the ROS-scavenger NAC. These data indicate a direct relationship between BCNU-induced red/ox imbalance and cyclin D1 down-modulation.

Then, we assessed whether D-cyclins levels were modulated by EC exposure to ischemia, a condition associated with increased ROS formation and oxidative stress. Eight hours of *in vitro* simulated ischemia induced a threefold decrease of cyclin D1 levels ($P<0.001$); this phenomenon was prevented by cell treatment with ROS scavengers.

We previously reported that hind-limb ischemia is associated with a sharp increase of oxidative stress. Thus, unilateral hind-limb ischemia was induced by femoral artery dissection and the D-cyclin expression in adductor muscle was assessed by immunohistochemistry and Western blotting after 8 h. Although cyclin D1 was barely detectable, cyclin D3 was clearly expressed in both EC and myofibers. In ischemic muscles, cyclin D3 expression levels decreased to 49.8±6.8% of the control ($P<0.001$). We previously demonstrated that in $p66^{ShcA}$ null mice, induction of ischemia by femoral artery excision does not increase oxidative stress. Likewise, $p66^{ShcA}$ deletion prevented cyclin D3 down-modulation induced by ischemia.

Thus, D-cyclins expression is down-modulated by a variety of stimuli inducing oxidative stress. For the sake

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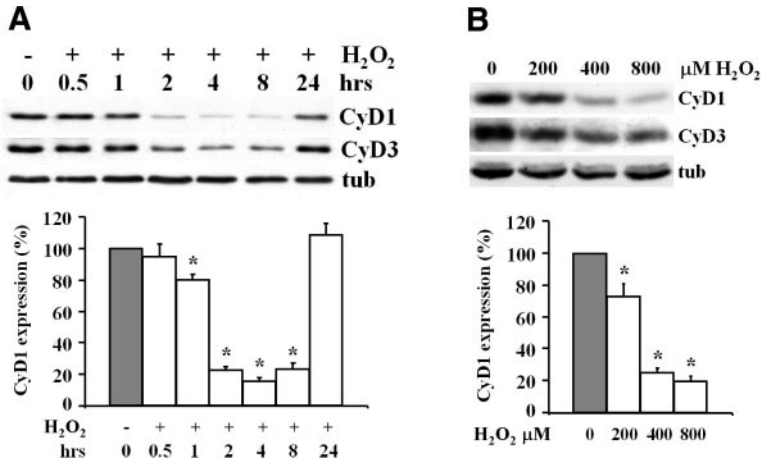


**Figure 1.** $H_2O_2$ treatment induces D-cyclins down-modulation. *A)* HUVEC were treated with 400 μmol/l $H_2O_2$ for the indicated time, followed by Western blotting. Graph shows cyclin D1 protein expression normalized for α-tubulin levels and expressed as % of untreated cells (*$P<0.001$ vs. control; $n=6–16$). *B)* Dose-dependent down-modulation of D-cyclins. HUVEC were incubated for 2 h with $H_2O_2$, followed by immunoblots. Graph shows cyclin D1 protein expression levels (*$P<0.001$ vs. control; $n=3–7$).

of simplicity, the molecular mechanism underlying this event was investigated only in $H_2O_2$-treated EC.

### 2. Cyclin D1 is degraded by the ubiquitin-protease pathway after $H_2O_2$ treatment

Northern blotting analysis showed that cyclin D1 mRNA was present throughout the time course of $H_2O_2$ treatment. Therefore, we analyzed whether $H_2O_2$ increased cyclin D1 protein turnover. Pulse-chase analysis of $^{35}$S-labeled cells revealed that after cell treatment with 600 μmol/l $H_2O_2$ for 2 h, cyclin D1 degradation rate almost doubled.

To assess proteasome involvement, HUVEC were treated with either lactacystin or *N*-acetyl-L-leucinyl-L-leucinyl-*N*-norleucinal (LLnL). These two cell permeant inhibitors of the proteasome completely prevented $H_2O_2$-induced down-modulation of cyclin D1. Then, we investigated whether $H_2O_2$ treatment induced cyclin D1 polyubiquitination. U2OS cells were treated with either LLnL alone or LLnL and $H_2O_2$. Afterward, cyclin D1 was immunoprecipitated, followed by immunoblotting to ubiquitin. $H_2O_2$ treatment induced an increase of the slower-migrating polyubiquitinated forms of cyclin D1.

### 3. Cyclin D1 degradation induced by oxidative stress is phospholipase C dependent

Since oxidants trigger phospholipase C (PLC)-γ activation, we tested whether PLC-γ was involved in $H_2O_2$-dependent degradation of cyclin D1. We found that PLC-γ activity was necessary to induce cyclin D1 degradation by $H_2O_2$. Indeed, HUVEC treatment with U73122, a selective inhibitor of PLC function, completely prevented $H_2O_2$-induced cyclin D1 down-modulation.

Moreover, PLC-γ activation was also sufficient to trigger cyclin D1 degradation in the absence of $H_2O_2$. HUVEC treatment with the PLC activator 2,4,6-trimethyl-*N*-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS) caused a dose-dependent down-modulation of cyclin D1 that was prevented by U73122 treatment.

### 4. $H_2O_2$-induced cyclin D1 degradation is $Ca^{2+}$ and CaMK dependent

PLC-γ activity catalyzes the generation of IP3 messenger that, in turn, binds to specific receptors (IP3R) on the ER and provokes an increase of intracellular calcium concentration ($[Ca^{2+}]_i$).

Kinetics of $Ca^{2+}$ mobilization measured in Fura-2-loaded cells showed that $H_2O_2$ treatment induced a steady $[Ca^{2+}]_i$ increase that correlated with cyclin D1 degradation dynamic. We found that cyclin D1 degradation was $[Ca^{2+}]_I$ dependent, since the intracellular $Ca^{2+}$ chelator BAPTA-AM prevented cyclin D1 down-modulation induced by $H_2O_2$ ($P<0.017$). In keeping with these results, HUVEC treatment with either 2-aminoethoxydiphenyl borate (2-APB) or xestospongin C, two IP3R inhibitors, significantly attenuated cyclin D1 down-modulation induced by $H_2O_2$.

CaMK transduces $[Ca^{2+}]_i$ elevation signals to a number of target proteins. Thus, we assayed whether CaMK activity was modulated after $H_2O_2$ stimulation. In keeping with previous data, HUVEC treatment with 400 μmol/l $H_2O_2$ for 30 min induced CaMK activity more than fivefold. We also found that CaMK activity was necessary to induce cyclin D1 degradation, since HUVEC treatment with CaMK inhibitor KN93 prevented $H_2O_2$-induced cyclin D1 down-modulation.

Finally, we asked whether forced expression of activated CaMK was sufficient to induce cyclin D1 degradation. In HUVEC overexpressing a constitutively active form of CaMKII, cyclin D1 down-modulation was induced. This negative regulation was due to degradation, since it was prevented by treatment with proteasome inhibitors.

### 5. Cyclin D1 overexpression increases cell susceptibility to $H_2O_2$-induced apoptosis

To investigate the physiological relevance of cyclin D1 down-modulation, we assessed whether override of cy-

clin D1 down-regulation via its forced overexpression affected cell proliferation and apoptosis in response to 400 μmol/l $H_2O_2$. HUVEC were infected with adenoviruses encoding either cyclin D1 (Ad-CyD1) or GFP (Ad-GFP, negative control), and both cell cycle phase distribution and apoptotic cell death were quantified by propidium iodide staining. Apoptosis was also determined measuring the amount of DNA apoptotic fragmentation.

On $H_2O_2$ treatment, the override of cyclin D1 down-modulation caused no significant differences in cell cycle. Conversely, Ad-CyD1-infected cells displayed significantly higher apoptosis after $H_2O_2$ incubation ($P<0.001$).

S-phase cells display increased sensitivity to $H_2O_2$. To test the role of cyclin D1 in cells undergoing DNA synthesis, HUVEC were synchronized in early S phase by aphidicolin block. Afterward, cells were allowed to re-enter S phase and treated with 400 μmol/l $H_2O_2$. We found that S-phase synchronization significantly enhanced apoptotic cell death induced by $H_2O_2$ in cyclin D1 overexpressing cells.

### 6. Overriding cyclin D1 degradation via CaMK inhibition enhances $H_2O_2$-induced apoptosis

To confirm the role of cyclin D1 down-modulation, we tested whether the override of cyclin D1 down-modulation elicited by CaMK inhibition increased cell sensitivity to $H_2O_2$-induced apoptosis (**Fig. 2**).

S-phase synchronized HUVEC were treated with $H_2O_2$, in the presence or absence of KN93 CaMK inhibitor. Treatment with 10 μmol/l KN93 significantly enhanced cell death induced by $H_2O_2$, as assessed measuring both the percentage of cells having subdiploid DNA content ($P<0.001$) and the apoptotic fragmentation of cellular DNA ($P<0.001$).

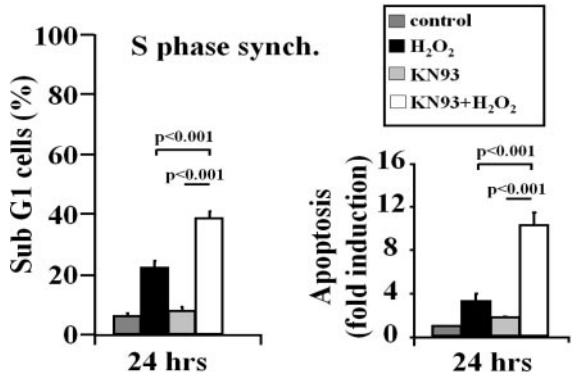


**Figure 2.** CaMK inhibition increases $H_2O_2$-induced apoptosis. S-phase synchronized HUVEC were treated with CaMK inhibitor KN93 (10 μmol/l) or solvent alone followed by incubation with or without 400 μmol/l $H_2O_2$ for additional 24 h. Apoptosis was measured by calculating percentage of cells displaying subdiploid DNA content (*left*, $n=11$) or the apoptotic DNA fragmentation (*right*, $n=4$). In cells incubated with KN93 and $H_2O_2$, apoptosis significantly increased over both cells treated with $H_2O_2$ alone or KN93 alone.

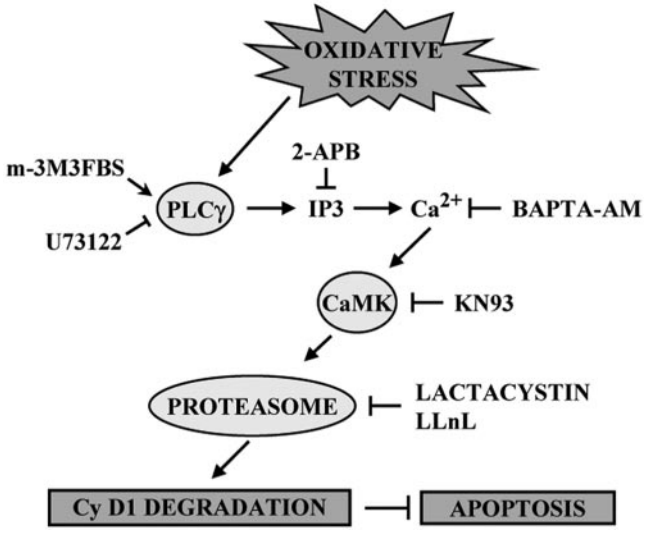


**Figure 3.** Diagram summarizing signal transduction pathway activated by $H_2O_2$ and leading to cyclin D1 degradation.

Then, we assessed whether this phenomenon was mediated by the deregulation of cyclin D1 levels. To this aim, KN93 and $H_2O_2$ were tested in cells where cyclin D1 expression was silenced via small interfering RNA (siRNA) strategy. Cyclin D1-siRNA significantly decreased cell sensitivity to $H_2O_2$ in KN93-treated cells ($P<0.001$), indicating that cyclin D1 down-modulation is the relevant target of CaMK inhibition.

## CONCLUSIONS AND SIGNIFICANCE

Understanding EC responses to oxidative stress may provide useful insights into aging mechanisms and into the pathogenesis of a variety of cardiovascular diseases. We found that cyclin D1 is rapidly down-modulated after EC treatment with sublethal doses of $H_2O_2$. This negative modulation is also observed after exposure to other oxidative stress-inducing stimuli.

To further investigate the molecular mechanism underpinning cyclin D1 down-modulation after $H_2O_2$ treatment, we found that $H_2O_2$ increased cyclin D1 ubiquitination followed by proteasome degradation. The study of the signaling pathways activated by oxidative stress showed that cyclin D1 degradation was dependent on PLC-IP3-mediated mobilization of intracellular $Ca^{2+}$ stores; $Ca^{2+}$ increase was in turn transduced by CaMK. Finally, the functional role of cyclin D1 degradation was examined. We found that overriding of cyclin D1 down-modulation via its forced overexpression or via CaMK inhibition increased cell sensitivity to apoptotic cell death induced by $H_2O_2$ (**Fig. 3**).

To the best of our knowledge, this is the first study linking together the posttranscriptional regulation of cyclin D1 levels after oxidative stress, the signaling involved in this phenomenon, and the functional consequences of cyclin D1 degradation. FJ

# Cyclin D1 degradation enhances endothelial cell survival upon oxidative stress

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ABSTRACT **The understanding of endothelial cell responses to oxidative stress may provide insights into aging mechanisms and into the pathogenesis of numerous cardiovascular diseases. In this study, we examined the regulation and the functional role of cyclin D1, a crucial player in cell proliferation and survival. On $H_2O_2$ treatment, endothelial cells showed a rapid down-modulation of cyclin D1. Other D-cyclins were similarly regulated, and this decrease was also observed after exposure to other oxidative stress-inducing stimuli, namely 1,3-bis (2 chloroethyl)-1 nitrosourea treatment and ischemia. $H_2O_2$ treatment induced cyclin D1 ubiquitination followed by proteasome degradation. Phospholipase C inhibition prevented cyclin D1 degradation, and its activation triggered cyclin D1 down-modulation in the absence of oxidative stress. Activated phospholipase C generates inositol-1,4,5-trisphosphate (IP3) and $Ca^{2+}$ release from internal stores. We found that both IP3-receptor inhibition and intracellular $Ca^{2+}$ chelation prevented cyclin D1 degradation induced by oxidative stress. Furthermore, $Ca^{2+}$ increase was transduced by $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK). In fact, $H_2O_2$ stimulated CaMK activity, CaMK inhibitors prevented $H_2O_2$-induced cyclin D1 down-modulation, and CaMK overexpression induced cyclin D1 degradation. Finally, overriding of cyclin D1 down-modulation via its forced overexpression or via CaMK inhibition increased cell sensitivity to $H_2O_2$-induced apoptotic cell death. Thus, cyclin D1 degradation enhances endothelial cell survival on oxidative stress.—Fasanaro, P., Magenta, A., Zaccagnini, G., Cicchillitti, L., Fucile, S., Eusebi, F., Biglioli, P., Capogrossi, M. C., Martelli, F. Cyclin D1 degradation enhances endothelial cell survival upon oxidative stress. *FASEB J.* 20, E503–E515 (2006)**

*Key Words: endothelium · ubiquitin-proteasome pathway · calcium signaling · apoptosis*

ENHANCED OXIDATIVE DAMAGE to endothelial cells (EC) is a prominent feature of many physiological and pathological conditions. Indeed, reactive oxygen species (ROS) have been shown to mediate EC apoptosis in a variety of cardiovascular diseases, including ischemia/reperfusion injury, diabetic vasculopathy, hypertension, atherosclerosis, and heart failure (1, 2). Therefore, it is of pivotal importance to understand ROS-activated mechanisms leading to EC apoptosis, as much as the adaptive responses inducing enhanced EC survival on oxidative stress.

Phospholipase C-γ (PLC-γ) has a prominent role in determining EC fate on ROS exposure (3). PLC-γ is activated by a mechanism relying on tyrosine phosphorylation after stimulation of growth factor receptor tyrosine kinases (4). Interestingly, several laboratories have demonstrated that PLC-γ undergoes tyrosine phosphorylation in response to oxidant exposure as well (3, 5, 6).

Activated PLC-γ cleaves the membrane phospholipid phosphatidylinositol-4,5-bisphosphate, generating two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The former activates protein kinase C (PKC) (7); the latter stimulates $Ca^{2+}$ release from the internal stores (8). Intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) regulates numerous physiopathological events, and the $Ca^{2+}$/calmodulin-dependent protein kinase family (CaMK) is frequently involved (9).

Several proteins that stimulate cells cycle progression have been demonstrated to induce apoptosis when deregulated. Specifically, the *cyclin D1* gene (*CCND1/BCL1/PRAD1*) plays an integral part in cell growth and survival control (10). D-type cyclins activate CDK4/6, which, in turn, phosphorylates pRb. This event triggers the derepression of a subset of proliferation-associated E2F target genes and induces the progression through the $G_1$ phase of the cell cycle (10).

Along with this role as a regulator of cell proliferation, increasing evidence indicates that cyclin D1 is a crucial player in certain cases of neuronal apoptosis (11). High levels of cyclin D1 expression also elicit apoptotis in non-neuronal cells, (12–14), indicating

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that uncoordinated cyclin D1 expression presents a survival challenge to the cell.

D-cyclins protein levels are tightly regulated at transcriptional and post-transcriptional concentration. Growth factor stimulation induces cyclin D1 mRNA synthesis (10). Moreover, cyclin D1 turnover is regulated by phosphorylation of specific threonine residues (Thr286 and Thr288), polyubiquitination, and subsequent degradation by the 26S proteasome (15). Thr286 phosphorylation of cyclin D1 is catalyzed by glycogen synthase kinase-3β (GSK-3β), on growth factor withdrawal (16) and by $p38^{Sapk2}$, after osmotic stress (17). Thr288 has been shown to be substrate of Mirk/dyrk1B kinase (18). Furthermore, alternative mechanisms of cyclin D1 ubiquitination have been described previously: a destruction box RxxL motif is necessary for cyclin D1 degradation induced by UV (19) and free-cyclin D1 (unbound to CDKs) is ubiquitinated independently of its phosphorylation on Thr286 and Thr288 (20).

Thus, although further studies are needed, it appears that different pathways are activated in different pathophysiological situations. In this study, we investigated how oxidative stress regulates cyclin D1 turnover and the functional relevance of cyclin D1 degradation for EC survival.

## MATERIALS AND METHODS

### Cell cultures

Human umbilical vein EC (HUVEC; Clonetics) and U2OS osteosarcoma cell line (American Type Culture Collection) were grown in EGM-2 (Bio-Whittaker) and Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, respectively (21, 22). Drug treatments, transfections, and simulated ischemia (23) are described in Supplementary Materials.

### Hind-limb ischemia

Surgical and immunohistochemical procedures are described in Supplementary Materials.

### Northern blot analysis

RNA extraction and Northern blotting were performed as described previously (22) using a specific cyclin D1 probe (20).

### Western blot analysis

Western blotting was performed as described previously (21, 23). Antibodies used are described in Supplementary Materials.

### Immunoprecipitation and pulse-chase analysis

Immunoprecipitation and pulse-chase experiments were performed as described previously (24) (see also Supplementary Materials).

### Calcium measurement

The variations of $[Ca^{2+}]_i$ were measured using a conventional fluorescence microscopy system as described previously (25; see also Supplementary Materials).

### CaMK assay

CaMK activity was measured using SignaTECT CaMK assay system (Promega) as described in Supplementary Materials.

### Cell cycle and apoptosis analysis

Cell cycle and apoptosis were measured using a Becton-Dickinson flow cytometer and CellQuest and ModFit softwares (21; see also Supplementary Materials). Alternatively, apoptosis was measured by cell death detection ELISA kit (Roche) according to manufacturer's instructions.

### Adenoviral infection

Adenoviral infection was performed as described previously (21; see also Supplementary Materials).

### Small interfering RNA-mediated gene silencing

Small interfering RNAs (siRNA) targeting cyclin D1 or CD4 (Santa Cruz) were transfected as described in Supplementary Materials.

### Statistical analysis

Variables were analyzed by Student's $t$ test and one-way ANOVA. A value of $P \leq 0.05$ was deemed statistically significant. Values are indicated ± SE.

## RESULTS

### D-cyclins are down-regulated in response to oxidative stress

To investigate cyclin D1 role in EC apoptosis on oxidative stress, HUVEC were treated with 400 μmol/l $H_2O_2$ for 1–24 h. **Figure 1*A*** shows that after 2 h of $H_2O_2$ treatment, cyclin D1 accumulation significantly decreased (23±2% of control, $P<0.001$) and proteins levels were maintained low until 8 h after treatment. Afterward, cyclin D1 protein started to recover and returned to control levels 24 h after $H_2O_2$ addition. Cyclin D3 protein was similarly modulated but to a lower extent (at 2 h it was 44±7% of the control, $P<0.001$), whereas cyclin D2 expression was not detectable.

In the adopted culture conditions, HUVEC treatment with 400 μmol/l induced negligible levels of cell death (21). We found that D-cyclins down-modulation was also observed after lethal doses (800 μmol/l) of $H_2O_2$ (21; Fig. 1*B*) and occurred with similar kinetics in other endothelial (bovine artery EC and porcine artery EC, not shown) and nonendothelial cell types (U2OS osteosarcoma cell line, see Supplementary Materials, Fig. S1*A*). Interestingly, U2OS expressed cyclin D2, which was negatively modulated by $H_2O_2$ as much as cyclin D1. It is worth noting that $H_2O_2$ concentrations >800 μmol/l $H_2O_2$ were necessary to induce cell death in U2OS (not shown), confirming that sublethal doses

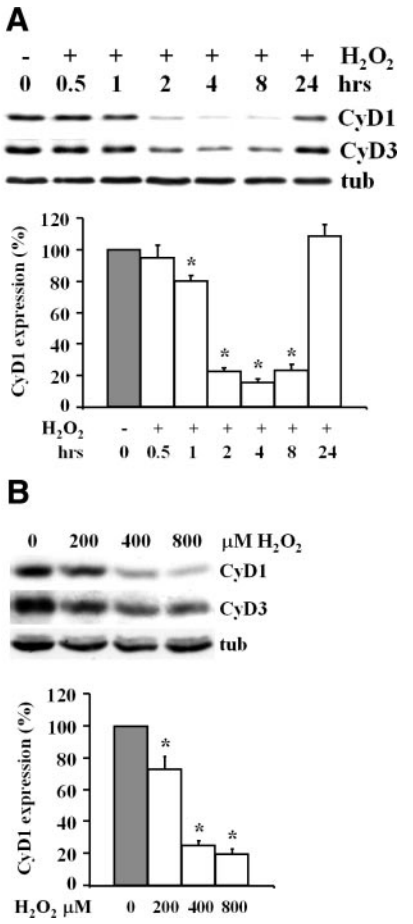


**Figure 1.** $H_2O_2$ treatment induces D-cyclin down-modulation. *A)* HUVEC were treated with 400 μmol/l $H_2O_2$ for indicated time, followed by Western blotting. Graph shows cyclin D1 protein expression normalized for α-tubulin levels and expressed as % untreated cells (*$P$<0.001 vs. control, $n$=6–16). *B)* Dose-dependent down-modulation of D cyclins. HUVEC were incubated for 2 h with $H_2O_2$, followed by immunoblots. Graph shows cyclin D1 protein expression levels (*$P$<0.001 vs. control; $n$=3–7).

of $H_2O_2$ are sufficient to trigger D-cyclins down-modulation.

Other cyclins were not modulated by oxidative stress: up to 8 h of treatment with 400 μmol/l $H_2O_2$ did not affect the expression of cyclins A, B, and E (see Supplementary Materials, Fig. S1*B*). Similarly, CDK 1, 2, 4, and 6 protein levels were not modulated, while $p21^{Waf1/Cip1/Sdi}$ was up-regulated 6–8 h after $H_2O_2$ treatment (not shown).

We also tested whether other interventions causing intracellular red/ox imbalance induced D-cyclin down-modulation.

EC are relevant targets of chemiotherapics (26). Specifically, the alkylating agent 1,3-bis (2 chloroethyl)-1 nitrosourea (BCNU, Carmustine), a widely used anticancer chemotherapy drug, is an inhibitor of glutathione (GSH) reductase that blocks the conversion of oxidized to reduced GSH (27). Incubation of HUVEC with 0.25 mmol/l BCNU for 2 h caused cyclin D1 down-regulation, and this phenomenon was prevented by preincubation with 10 mmol/l NAC. Treatment with NAC prevents the decrease of reduced GSH induced by BCNU (27), indicating a direct relationship between BCNU induced red/ox imbalance and cyclin D1 down-modulation (**Fig. 2*A***). We found that cyclin D3 was similarly regulated.

We also tested whether D-cyclin levels were modulated on EC exposure to ischemia, a condition associated with increased ROS formation and oxidative stress ((23) and references therein). We found that 8 h of simulated ischemia induced a threefold decrease of cyclin D1 levels ($P$<0.001), and this phenomenon was prevented by cell treatment with catalase and 4-hydroxy-TEMPO ROS scavengers (Fig. 2*B*). Similar results were obtained with cyclin D3.

To assess whether D-cyclin expression was also regulated by ischemia in vivo, we tested their expression in a mouse model of hind-limb ischemia (23). Unilateral hind-limb ischemia was induced by femoral artery removal, and adductor muscles were analyzed after 8 h. In keeping with previous results (23), femoral artery removal induced a 5.4 ± 0.5 increase of apoptosis, as assessed by TUNEL assay (see Supplementary Materials, Fig. S2). D-cyclins expression was assessed by immunohistochemistry in adductor muscle sections: Whereas cyclin D1 expression was barely detectable (not shown), cyclin D3 antibody (Ab) clearly marked EC nuclei in normoperfused mice (Fig. 2*C*). Some nuclei of myofibers were positively stained as well. In ischemic muscles, cyclin D3 expression decreased in both EC and myofibers. This event was quantified by Western blotting of normoperfused and ischemic muscles (Fig. 2*D*). On ischemia, cyclin D3 levels were 49.8 ± 6.8% of the control ($P$<0.001). To assess whether cyclin D3 down-modulation was indeed due to ischemia-induced oxidative stress, we took advantage of $p66^{ShcA}$ null (−/−) mouse model. Femoral artery excision induces a similar decrease of blood perfusion in $p66^{ShcA}$ −/− and wt mice. However, oxidative stress is not increased when $p66^{ShcA}$ −/− mice are exposed to ischemia (23). Figure 2*D* shows that $p66^{ShcA}$ deletion prevented cyclin D3 down-modulation induced by ischemia.

We concluded that D-cyclin expression is down-modulated by a variety of oxidative stress inducing stimuli. The molecular mechanisms underlying this event were investigated in $H_2O_2$-treated cells for the sake of simplicity.

### $H_2O_2$ induces cyclin D1 degradation

Northern blotting analysis showed that cyclin D1 mRNA was present throughout the time course of $H_2O_2$ treatment (see Supplementary Materials, Fig. S3). Therefore, we analyzed whether $H_2O_2$ increased cyclin D1 protein turnover. Easily transfectable U2OS cells were chosen as experimental system. A plasmid encoding hemagglutinin (HA) epitope-tagged human cyclin D1 was transfected, and cells

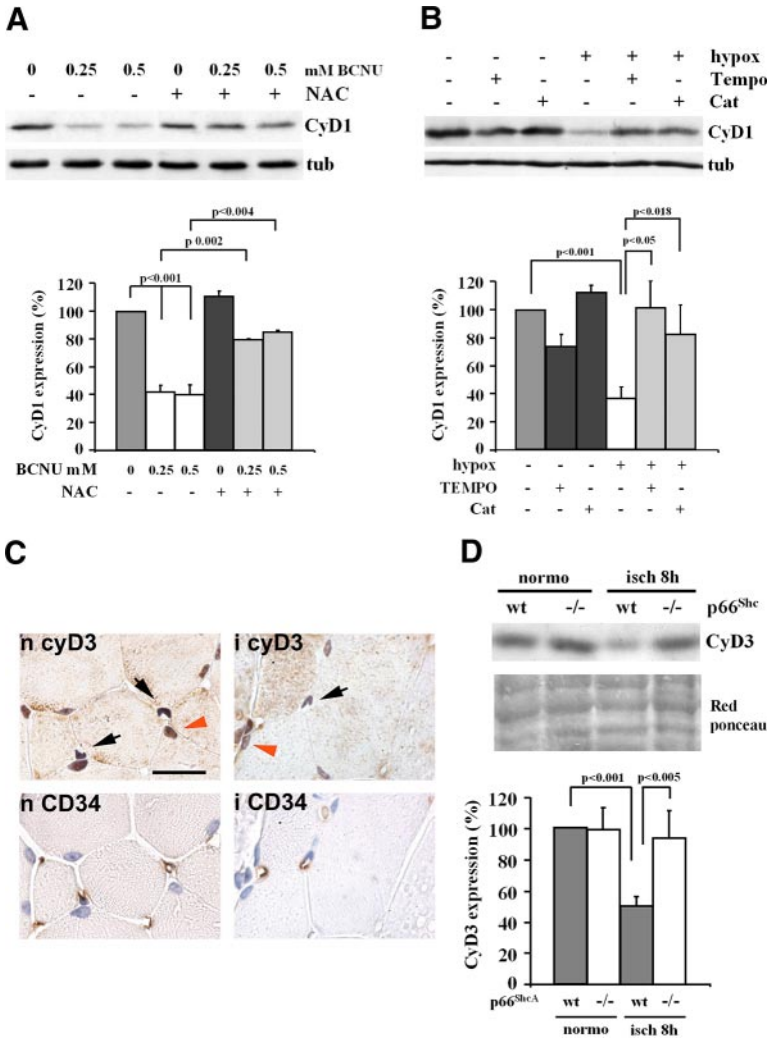


**Figure 2.** Oxidative stress induces D-cyclin down-modulation. *A)* BCNU induces cyclin D1 down-modulation. HUVEC were either preincubated with 10 mmol/l NAC (+) or sham treated (-) for 30 min, followed by incubation with indicated concentrations of BCNU for 2 h and immunoblotting. Graph shows cyclin D1 protein expression levels ($n$=3). *B)* HUVEC were incubated for 8 h with glucose-free medium in normoxic conditions or in an hypoxic camera (hypox) in the presence or absence of 5000 U/ml of catalase (cat) or 2.5 mmol/l 4-hydroxy-TEMPO, followed by immunoblots. Graph shows cyclin D1 protein expression levels ($n$=3). *C)* Ischemia induces cyclin D3 down-modulation. Representative cyclin D3 immunostainings (*top*) of normoperfused (n) and ischemic (i) adductor muscles. EC were identified in serial sections by CD34 immunostaining (*bottom*). Magnification ×1000; bar = 15 μm. Black arrowheads indicate cyclin D3 positive nuclei of EC, and red arrowheads indicate cyclin D3 positive nuclei most likely belonging to myofibers. *D)* Representative ($n$=5–9) Western blotting of adductor muscle extracts derived from normo-perfused (normo) or ischemic (isch 8h) $p66^{ShcA}$ wt or -/- mice, 8 h after femoral artery dissection. Cyclin D3 was detected using a specific Ab. Red Ponceau staining indicates uniform protein loading.

were treated with 600 μmol/l $H_2O_2$ for 2 h. Thereafter, they were pulse labeled for 30 min with [$^{35}$S]methionine and chased with medium containing cold methionine. **Figure 3** shows that the turnover of both ectopically expressed (black arrow) and endogenous (gray arrow) cyclin D1 significantly increased in pro-oxidant conditions.

Pharmacological alterations in the glycosylation machinery or in calcium levels can disrupt normal endoplasmic reticulum (ER) protein biogenesis and trigger a complex chain of events termed the unfolded protein response (UPR) (28). Activation of the mammalian UPR is characterized by increased transcription of a series of molecular chaperones, such as GRP78, as well as by the block of cyclin D1 translation (28).

No significant decrease of cyclin D1 translation was observed in our experimental conditions. Indeed, Fig. 3*A* shows that a similar rate of $^{35}$S-labeled cyclin D1 was expressed both in $H_2O_2$- and mock-treated cells.

Moreover, $H_2O_2$ treatment failed to induce GRP78 expression (see Supplementary Materials, Fig. S4).

We concluded that, on $H_2O_2$ treatment, cyclin D1 degradation was induced and that UPR played no major role in cyclin D1 negative regulation.

### Cyclin D1 is degraded by the ubiquitin-proteasome pathway after $H_2O_2$ treatment

To test whether cyclin D1 down-modulation induced by $H_2O_2$ could be prevented by proteasome inhibition, HUVEC were cultured in the presence of either lactacystin or *N*-acetyl-L-leucinyl-L-leucinyl-*N*-norleucinal (LLnL), two potent cell permeant inhibitors of the proteasome. Both lactacystin (**Fig. 4*A***) and LLnL (see Supplementary Materials, Fig. S5) completely prevented $H_2O_2$-induced down-modulation of cyclin D1. Conversely, cell treatment with *N*-acetyl-leucyl-leucyl-methioninal, a Calpain inhibitor, failed to do so (not shown). Similar results were obtained when lactacystin was added after $H_2O_2$ treatment (not shown), indicating that proteasome inhibition can not only prevent but can also revert cyclin D1 degradation induced by oxidative stress.

To further investigate cyclin D1 degradation, we tested whether cyclin D1 was polyubiquitinated in vivo. U2OS cells were treated with either LLnL alone or LLnL and $H_2O_2$. Then, cyclin D1 was immunoprecipitated followed by immunoblotting to ubiquitin (Fig. 4*B*, lanes 2 and 3). Figure 4*B* shows that slower-migrating

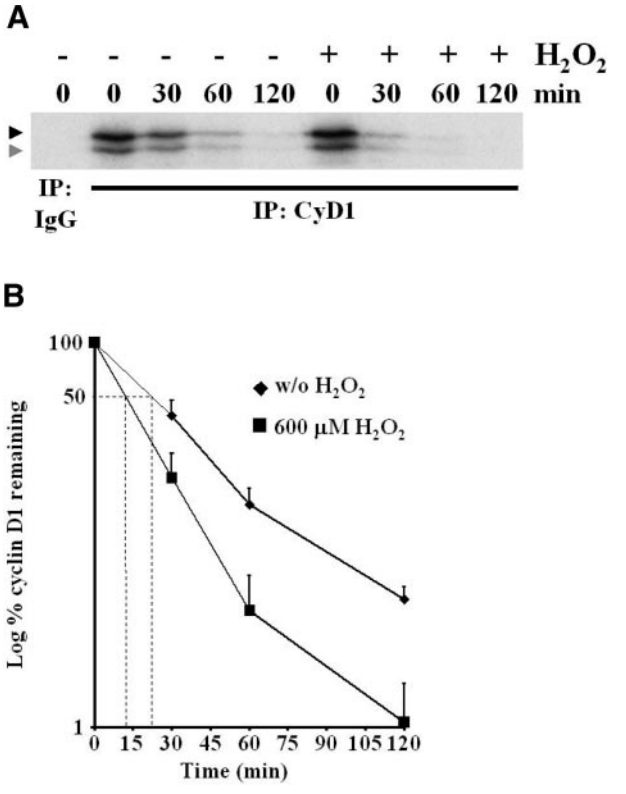


**Figure 3.** $H_2O_2$ induces cyclin D1 degradation. U2OS cells were transfected with a plasmid encoding HA-tagged human cyclin D1. Thirty six hours later, cells were treated with 600 μmol/l $H_2O_2$ for 2 h, pulse labeled with [$^{35}$S]methionine, and chased with medium containing cold methionine. Then, cells were lysed and immunoprecipitated with an Ab to cyclin D1 (CyD1) or to an irrelevant control (IgG), followed by SDS-PAGE. *A)* $H_2O_2$ induces an increase of turnover of both ectopically expressed (black arrow) and endogenous (gray arrow) cyclin D1. HA-tag caused an electrophoretic mobility slow-down of the overexpressed cyclin D1 allele. *B)* Cyclin D1 signals are expressed as % value at time 0. Dotted lines indicate cyclin D1 half-life.

polyubiquitinated forms of cyclin D1 were enriched in the immunoprecipitates derived from cells treated with LLnL and $H_2O_2$ (lane 3, brackets). The same extract immunoprecipitated with an irrelevant Ab failed to detect any specific signal (lane 1). Similar results were obtained with a different cyclin D1 Ab (not shown).

We concluded that the proteolysis of cyclin D1 on $H_2O_2$ treatment is controlled by the ubiquitin-proteasome pathway.

To assess whether cyclin D1 phosphorylation at Thr286 and Thr288 was necessary for cyclin D1 degradation after $H_2O_2$ treatment, a specific cyclin D1 allele bearing mutations of both sites (cyclin D1-mut) was assayed (20). U2OS cells overexpressing HA-tagged cyclin D1-mut showed a decrease of cyclin D1 accumulation comparable to that obtained with cyclin D1 wt after $H_2O_2$ treatment (**Fig. 5*A***). Similar results were obtained using mouse cyclin D1 mutated at Thr286 (15) (not shown).

These data suggest that cyclin D1 phosphorylation is not necessary for cyclin D1 demise induced by $H_2O_2$.

**Cyclin D1 degradation induced by oxidative stress is PLC dependent**

Oxidants trigger PLC-γ activation by a tyrosine phosphorylation dependent mechanism (3, 5, 6, and data not shown). Thus, we tested whether PLC-γ was involved in $H_2O_2$-dependent degradation of cyclin D1. To determine whether PLC-γ activity was necessary to

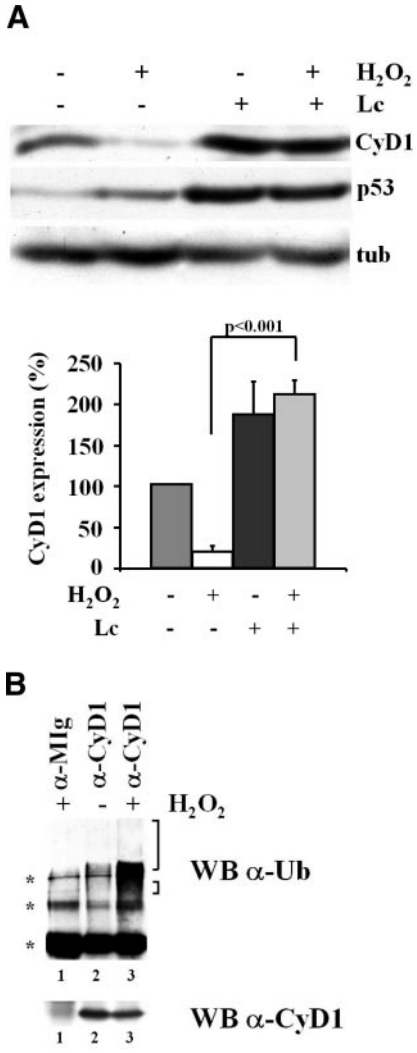


**Figure 4.** Cyclin D1 is degraded by ubiquitin-proteasome pathway. *A)* The proteasome inhibitor Lactacystin prevented $H_2O_2$-induced cyclin D1 down-modulation. HUVEC were incubated with 25 μmol/. lactacystin for 2 h. Then, $H_2O_2$ (400 μmol/l) was added and 2 h later cells were lysed and immunoblots were performed. Graph shows cyclin D1 protein expression levels ($n$=3). *B)* Cyclin D1 is polyubiquitinated on $H_2O_2$ treatment. U2OS were incubated with 50 μmol/l LLnL for 2 h. Afterward, cells were treated with 400 μmol/l $H_2O_2$ and 2 h later were lysed. Immunoprecipitations with irrelevant (α-MIg) or cyclin D1 specific Ab (α-CyD1) were performed, followed by Western blotting with a ubiquitin Ab (*top*). Asterisks indicate aspecific bands, probably due to immunoglobulins. Brackets indicate specific smears attributable to high molecular weight ubiquitinated species of cyclin D1. Then, membrane was stripped and reprobed with a cyclin D1 Ab to to assess equal efficiency of immunoprecipitation (*bottom*).

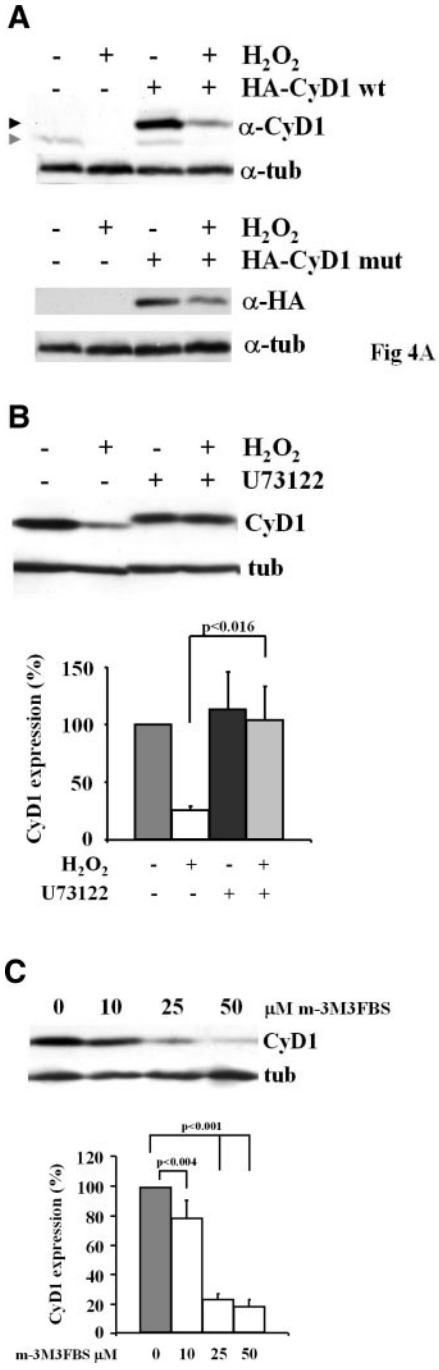


**Figure 5**. Cyclin D1 degradation is PLC-γ dependent. *A)* Thr286 and Thr288 are not necessary for cyclin D1 degradation. U2OS cells were transfected with a plasmids encoding HA-tagged cyclin D1 wt, HA-tagged cyclin D1 mutated at Thr286, and Thr288 (CyD1-mut) or with backbone plasmid. Thirty-six hours after transfection, cells were treated with 400 μmol/l $H_2O_2$ followed by immunoblot with antibodies to cyclin D1 (*top*) or HA-tag (*bottom*). In first case, HA-tag caused an electrophoretic mobility slow-down of the overexpressed cyclin D1 allele (black arrowhead) that allowed its separation from endogenous cyclin D1 (gray arrowhead). On $H_2O_2$ treatment, overexpressed cyclin D1 wt levels were 41 ± 0.2% of control ($P<0.015$; $n=3$); cyclin D1 mut levels were 62 ± 0.1% of control ($P<0.008$; $n=3$). Difference between overexpressed cyclin D1 wt and mut levels in $H_2O_2$ treated samples was not significant. *B)* PLC inhibition prevents cyclin D1 degradation induced by $H_2O_2$. HUVEC were incubated with 40 μmol/L U73122 for 10 min; then cells were treated with 400 μmol/l $H_2O_2$ for 2 h followed by immunoblotting. In our experimental conditions, U73122 treatment induced a minimal retard in electrophoretic mobility of cyclin D1 and, to a lower extent, of tubulin. Graph shows cyclin D1 protein expression levels ($n=3$). *C)* PLC activation triggers cyclin D1 degradation in the absence of $H_2O_2$. HUVEC were treated with indicated doses of m-3M3FBS for 2 h, followed by immunoblotting. Graph shows cyclin D1 protein expression levels ($n=3$–6).

induce cyclin D1 degradation by $H_2O_2$, HUVEC were pretreated with a selective inhibitor of phosphoinositide-specific PLC function, U73122. Figure 5*B* shows that U73122 completely prevented $H_2O_2$-induced cyclin D1 down-modulation. The inactive analog of U73122, known as U73433, was not assayed because of its toxicity in the experimental conditions used, possibly due to activities unrelated to PLC inhibition (6).

Then, we tested whether PLC-γ activation was sufficient to trigger cyclin D1 degradation in the absence of $H_2O_2$. To this aim, we used 2,4,6-trimethyl-*N*-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS), a compound known to directly activate all PLC isoforms (29). HUVEC treatment with m-3M3FBS caused a dose-dependent down-modulation of cyclin D1 (Fig. 5*C*). As expected, U73122 prevented m-3M3FBS induced cyclin D1 negative modulation (not shown).

Although a role of other PLC isoforms cannot be ruled out, the most likely interpretation of these results is that $H_2O_2$-dependent degradation of cyclin D1 is mediated by PLC-γ.

### $H_2O_2$-induced cyclin D1 degradation is $Ca^{2+}$ dependent

PLC-γ activity catalyzes the generation of IP3 and DAG second messengers (3, 4): The former binds to specific receptors (IP3R) on the ER and provokes an increase of $[Ca^{2+}]_i$ (8), whereas the latter is a direct activator of PKC (7). HUVEC treatment with Go6850, calphostin C, and H-7 dihydrochloride, three PKC inhibitors (7), failed to prevent cyclin D1 down-modulation induced by $H_2O_2$ (not shown). Moreover, HUVEC treatment with phorbol 12-myristate 13-acetate (PMA), a specific activator of PKC (7), at concentrations ranging from 10 nmol/l to 50 μmol/l for 2 h, did not trigger cyclin D1 degradation (not shown). These data strongly suggest that PKC is not involved in $H_2O_2$-induced cyclin D1 degradation.

Then, we determined whether $Ca^{2+}$ mobilization was involved in cyclin D1 degradation induced by $H_2O_2$.

To assess whether $H_2O_2$ and m-3M3FBS treatment induced an increase of $[Ca^{2+}]_i$ in the adopted experimental conditions, Fura 2-acetoxymethyl ester loaded cells were assayed using time-resolved fluorescent microscopy (25). HUVEC incubation with both 400 and 800 μmol/l $H_2O_2$ induced a slow developing rise of $[Ca^{2+}]_i$ that started ~10 min after $H_2O_2$ addition and lasted more than 40 min from the beginning of the

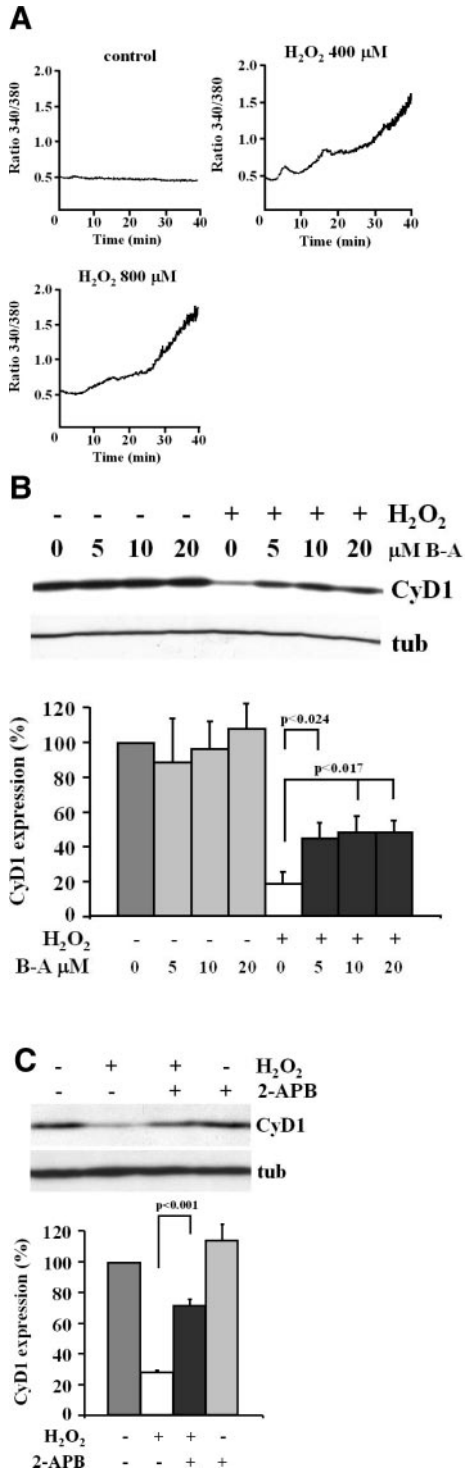


**Figure 6.** $H_2O_2$-induced cyclin D1 degradation is $Ca^{2+}$ dependent. *A)* $H_2O_2$ treatment induces a slow developing increase in $[Ca^{2+}]_i$. HUVEC were loaded with FURA 2-acetoxymethyl ester and $[Ca^{2+}]_i$ variations were measured using time-resolved fluorescent microscopy. Concentration of $[Ca^{2+}]_i$ was expressed as the time-resolved ratio between fluorescence images obtained at 340 and 380 nM excitation wavelengths. A representative trace for both $H_2O_2$ concentrations is shown. *B)* $H_2O_2$-induced cyclin D1 degradation is $Ca^{2+}$ dependent. HUVEC were incubated for 30 min with the intracellular $Ca^{2+}$-chelator BAPTA-acetoxymethyl ester and then incubated with 400 μmol/l $H_2O_2$ for 2 h. Afterward, cells were harvested and immunoblottings performed. Graph shows cyclin D1 protein expression levels ($n$=3). *C)* IP3Rs inhibitor prevents cyclin D1 down-modulation induced by $H_2O_2$. HUVEC were pretreated for 10 min with 300 nmol/l 2-APB. Then, $H_2O_2$ (400 μmol/l) was added and 2 h later immunoblots were performed. Graph shows cyclin D1 protein expression levels ($P$<0.001; $n$=3).

experiment (**Fig. 6*A***). These results are in agreement with previous reports showing that, while EC exposure to low doses of $H_2O_2$ triggered repetitive $[Ca^{2+}]_i$ oscillations, $H_2O_2$ >500 μmol/l induced a steady $[Ca^{2+}]_i$ increase without oscillations (30). Cell treatment with 25 μmol/l m-3M3FBS induced a rapid increase of $[Ca^{2+}]_i$ that was sustained throughout the time course (see Supplementary Materials, Fig. S6).

Then, it was assessed whether $H_2O_2$-induced cyclin D1 degradation was $[Ca^{2+}]_i$ dependent. To this aim, we tested whether cell treatment with BAPTA-AM, an intracellular $Ca^{2+}$ chelator, prevented cyclin D1 down-modulation. Figure 6*B* shows that BAPTA-AM significantly prevented cyclin D1 down-modulation induced by $H_2O_2$ (at 10 and 20 μmol/l, $P$<0.017).

To gain further insight into $[Ca^{2+}]_i$ role in cyclin D1 degradation, HUVEC were pretreated with either 2-aminoethoxydiphenyl borate (2-APB) or xestospongin C, two IP3Rs inhibitors, before incubation with $H_2O_2$. Both 2-APB (Fig. 6*C*) and xestospongin C (see Supplementary Materials, Fig. S7) significantly attenuated cyclin D1 down-modulation induced by $H_2O_2$.

In conclusion, although it is possible that other pathways contribute to cyclin D1 degradation induced by $H_2O_2$, the present results demonstrated that $H_2O_2$-induced $[Ca^{2+}]_i$ increase plays a pivotal role in cyclin D1 degradation.

### $H_2O_2$-induced cyclin D1 degradation is CaMK dependent

CaMK transduces $[Ca^{2+}]_i$ elevation signals to a number of target proteins (9). Thus, we assayed whether CaMK activity was modulated after $H_2O_2$ stimulation. Thapsigargin, a SERCA inhibitor that elicits $[Ca^{2+}]_i$ increase (8), was used as a positive control. In keeping with previous data (31), after HUVEC incubation with 400 μmol/l $H_2O_2$, CaMK activity was strongly enhanced up to 1 h and declined later on (**Fig. 7*A***). As expected, HUVEC treatment with 25 μM m-3M3FBS stimulated CaMK activity as well (see Supplementary Materials, Fig. S8).

Then, we assessed whether CaMK activity was necessary to induce cyclin D1 degradation. We found that HUVEC treatment with the CaMK inhibitor KN93 prevented $H_2O_2$-induced cyclin D1 down-modulation (Fig. 7*B*). Similar results were obtained with phenoxybenzamine, a calmodulin (CaM) inhibitor, whereas KN92, an inactive analog of KN93, was inert (not shown).

Finally, we asked whether forced expression of activated CaMK was sufficient to induce cyclin D1 degra-

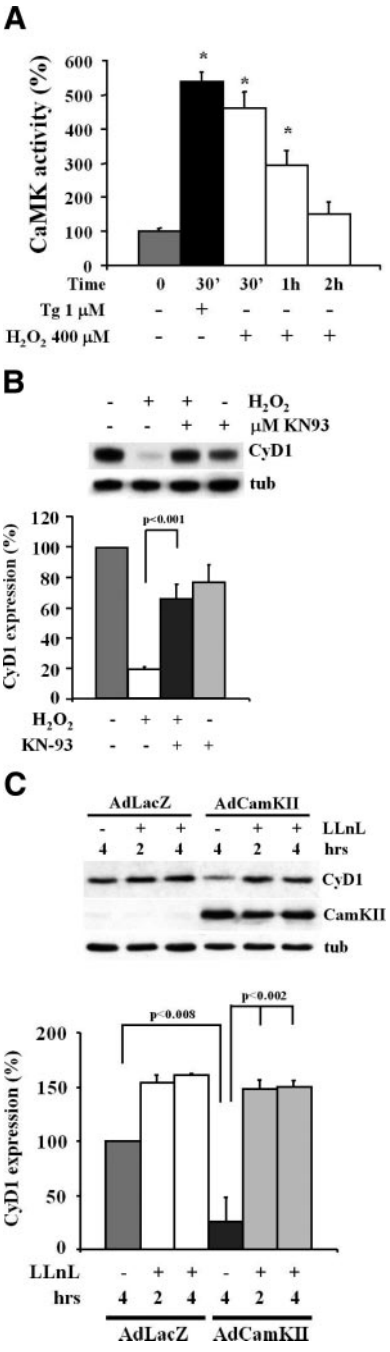


**Figure 7.** CaMK plays a crucial role in $H_2O_2$-induced cyclin D1 degradation. *A)* CaMK activity was positively modulated after $H_2O_2$ stimulation. HUVEC were treated either with 1 μmol/l thapsigargin (Tg, positive control) or with 400 μmol/l $H_2O_2$ for indicated periods of time. CaMK activity was expressed as % untreated cells (*$P<0.002$). *B)* CaMK inhibitor KN93 prevented cyclin D1 degradation induced by $H_2O_2$. HUVEC were incubated with 10 μmol/l KN93 for 30 min; then, cells were treated with 400 μmol/l $H_2O_2$ for 2 h followed by immunoblotting. Graph shows cyclin D1 protein expression levels ($n=4$). *C)* Overexpression of constitutively activated CaMK is sufficient to induce cyclin D1 degradation. HUVEC were infected with 50 MOI of adenoviruses encoding either CaMKII-D3 (AdCaMK) or LacZ (AdLacZ). Fourteen hours later, cells were incubated with 50 μmol/l LLnL or solvent alone for 2 or 4 h, followed by immunoblotting. Graph shows cyclin D1 protein expression levels ($n=4$).

dation. HUVEC were infected with a recombinant replication-defective adenovirus expressing a CaMKII allele that is locked into the activated state by site-directed mutagenesis (CaMKII-D3) (32). Although the overexpression of a control protein (LacZ) was inert, CaMKII-D3 overexpression triggered cyclin D1 down-modulation. This negative regulation was due to proteasomal degradation, since it was prevented by treatment with both LLnL (Fig. 7*C*) and lactacystin (not shown).

Although the adopted experimental approach does not allow us to distinguish among the different CaMK isoforms, the present results demonstrate that CaMK plays a crucial role in cyclin D1 degradation.

The ERK arm of the MAPK signaling pathway plays an important role in cell survival control. Increase of $[Ca^{2+}]_i$ promote ERK activation either directly (33, 34) or by the intervention of CaMK (35, 36). We found that HUVEC treatment with as much as 50 μM PD038059, a specific ERK inhibitor, did not prevent $H_2O_2$-induced cyclin D1 down-modulation. These data indicate that ERK is not necessary for this event (see Supplementary Materials, Fig. S9).

### Cyclin D1 overexpression increases cell susceptibility to $H_2O_2$-induced apoptosis

We attempted to investigate the physiological relevance of cyclin D1 down-modulation. To this aim, we assessed whether the override of cyclin D1 down-regulation via its forced over-expression affected cell proliferation and apoptosis in response to $H_2O_2$.

HUVEC were infected with adenoviruses encoding either cyclin D1 (AdCyD1) or GFP (AdGFP), as a negative control. Then, cells were treated with 400 μmol/l $H_2O_2$ or solvent alone and both cell cycle phase distribution and apoptotic cell death were quantified in propidium iodide stained cells. Cyclin D1 over-expression was demonstrated by Western blot analysis (see Supplementary Materials, Fig. S10*A*). Interestingly, the higher levels of over-expression achieved with adenoviral vectors were sufficient to overcome cyclin D1 degradation, yielding a complete override of cyclin D1 down-modulation on $H_2O_2$ treatment.

We found that $H_2O_2$ treatment increased the portion of cells in $G_2/M$ phases and inhibited DNA synthesis. However, no significant difference was found after override of cyclin D1 down-modulation (see Supplementary Materials, Fig. S10*B* and *C*).

Then, cell death was assessed calculating the percentage of cells displaying subdiploid DNA content (**Fig. 8*A***). In untreated AdGFP-infected cells, the apoptotic subdiploid fraction was less than 4.5% at 12 and 24 h; at 48 h some cell death was present (14.2±0.6%), possibly as a secondary effect of adenoviral infection. At this $H_2O_2$ dosage, no significant increase in the subdiploid fraction was observed within 48 h of $H_2O_2$ treatment. As previously reported (11–14), cyclin D1 overexpression in the absence of $H_2O_2$ increased the percentage of cells exhibiting subdiploid DNA content ($P<0.001$).

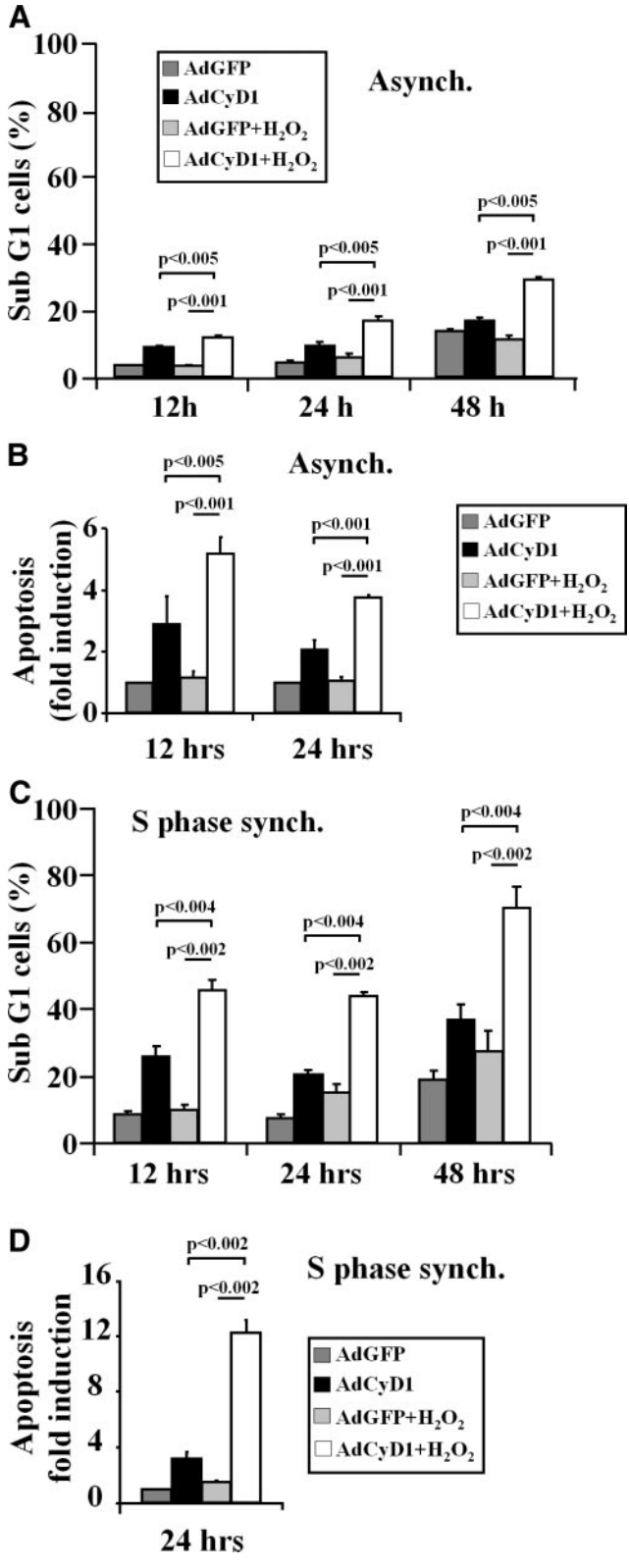


**Figure 8.** Cyclin D1 over-expression increases cell susceptibility to $H_2O_2$-induced apoptosis. Asynchronously growing HUVEC were infected with 50 MOI of adenoviruses encoding either cyclin D1 (AdCyD1) or GFP (AdGFP). Eighteen hours after infection, 400 μmol/l $H_2O_2$ or solvent alone were added and apoptosis rates were determined at the indicated times ($n$=3). *A)* % Propidium iodide stained cells displaying subdiploid DNA content was evaluated by flow cytometry. Alternatively (*B*), apoptosis was measured determining fragmentation of cellular DNA. After $H_2O_2$ incubation, apoptosis significantly increased over both untreated AdCyD1 infected cells ($P$<0.005) and Ad-GFP infected cells treated with $H_2O_2$ ($P$<0.001). *C, D)* HUVEC were infected with AdCyD1 or AdGFP, synchronized in early S phase and treated with either 400 μmol/l $H_2O_2$ or solvent alone for the indicated times. Apoptosis was measured ($n$=4) calculating cell %displaying subdiploid DNA content (*C*) or apoptotic fragmentation of cellular DNA (*D*). As expected, $H_2O_2$ treatment induced a small but significant ($P$<0.03) increase of apoptosis in AdGFP-infected cells. More importantly, apoptosis of $H_2O_2$-treated AdCyD1-infected cells significantly increased over both untreated cells ($P$<0.004) and Ad-GFP infected cells treated with $H_2O_2$ ($P$<0.002).

After $H_2O_2$ incubation, however, the apoptotic subdiploid fraction was 17.5±1.3% and significantly higher than both untreated cyclin D1 overexpressing cells ($P$<0.005) and AdGFP-infected cells treated with $H_2O_2$ ($P$<0.001).

To confirm that the observed increase of the subdiploid fraction was due to apoptosis, the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA was assessed (Fig. 8*B*). Since this is an earlier parameter compared to the appearance of the subdiploid population, the assay was conducted at 12 and 24 h after $H_2O_2$ treatment. Only minimal levels of nucleosomal fragmented DNA were found in both $H_2O_2$-treated and -untreated cells infected with AdGFP. In contrast, in cyclin D1 overexpressing cells, apoptosis significantly increased after treatment with $H_2O_2$ over both untreated cyclin D1 overexpressing cells ($P$<0.007) and AdGFP-infected cells treated with $H_2O_2$ ($P$<0.001).

S-phase cells display an exquisitely high sensitivity to $H_2O_2$ (37, 38). Thus, one may expect that the override of cyclin D1 down-modulation may have particularly dramatic effects in cells undergoing DNA synthesis. To test this hypothesis, cyclin D1 or GFP overexpressing HUVEC were synchronized in early S phase by aphidicolin and then released. With the use of this synchronization procedure, S-phase fraction increased ~7 fold (AdGFP, from 6.8±0.4% to 52.9±3.1%, $P$<0.001; AdCyD1 from 8.3±1.1 to 56.6±4.0%, $P$<0.001). S-phase cells were then treated with either $H_2O_2$ or solvent alone for 12, 24, or 48 h, and apoptosis was measured calculating the percentage of cells displaying subdiploid DNA content by flow cytometry (Fig. 8*C*). S-phase synchronization enhanced cell death induced by $H_2O_2$ in control cells and even more significantly in cyclin D1 overexpressing cells: indeed, 24 h after treatment 43.9 ± 1.0% of the cells showed subdiploid DNA content. Similar results were obtained measuring the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA (Fig. 8*D*).

In conclusion, overcoming cyclin D1 down-modulation by its overexpression makes cells more sensitive to $H_2O_2$-induced apoptosis.

### Overriding cyclin D1 degradation via CaMK inhibition enhances $H_2O_2$-induced apoptosis

We asked whether the override of cyclin D1 down-modulation elicited by CaMK inhibition increased cell sensitivity to $H_2O_2$-induced apoptosis.

HUVEC were synchronized in early S phase and then treated for 24 h with $H_2O_2$, in the presence or absence of CaMK inhibitor KN93. **Figure 9*A*** shows that KN93 treatment significantly enhanced cell death induced by $H_2O_2$, as assessed calculating both the percentage of cells that had subdiploid DNA content (Fig. 9*A*, *left*, $P<0.001$) and measuring the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA (Fig. 9*A*, *right*, $P<0.001$).

To assess whether this phenomenon was mediated by the deregulation of cyclin D1 levels, cell sensitivity to KN93 and $H_2O_2$ was tested in cells where cyclin D1 expression was silenced via siRNA strategy. U2OS cells were transfected with either control or cyclin D1-specific siRNA, yielding a 64.3±4.7% inhibition of cyclin D1 expression ($P<0.001$). To achieve optimal levels of siRNA transfection, cells were seeded at low density. Cell density has been shown to be a crucial parameter for cell viability: thus, to minimize toxicity, cells were not synchronized. Figure 9*B* shows that $H_2O_2$ treatment of low-density cultures of U2OS induced a 2.1 ± 0.4-fold increase of apoptosis that was further increased (3.5±0.1-fold) by the CaMK inhibitor KN93. We found that cyclin D1-specific siRNA decreased cell sensitivity to $H_2O_2$ in KN93 treated cells ($P<0.001$), indicating that cyclin D1 down-modulation is the relevant target of CaMK inhibition.

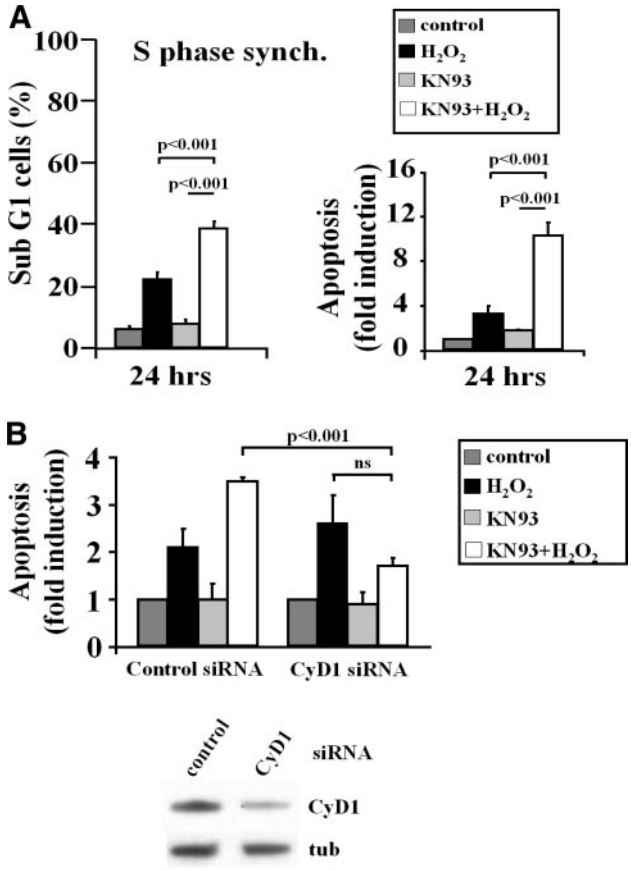


**Figure 9.** CaMK inhibition increases $H_2O_2$-induced apoptosis. *A)* HUVEC were synchronized in early S phase and treated with either CaMK inhibitor KN93 (10 μmol/l) or solvent alone. One hour later, $H_2O_2$ or solvent alone were added and cells were incubated for additional 24 h. Apoptosis was measured calculating % of cells displaying subdiploid DNA content by flow cytometry (*left*, $n=11$) or apoptotic DNA fragmentation (*right*, $n=4$). Effect of KN93 alone was negligible, while as expected in synchronized cells, $H_2O_2$ treatment induced a 3-fold increase of apoptosis. In cells incubated with KN93 and $H_2O_2$, apoptosis significantly increased over both cells treated with $H_2O_2$ alone or KN93 alone. *B)* $H_2O_2$ sensitivity induced by CaMK inhibition is mediated by the deregulation of cyclin D1 levels. Low density cultures of U2OS cells were transfected with either CD4 (negative control) or cyclin D1-specific siRNAs. After 18 h, cells were treated with either CaMK inhibitor KN93 (10 μmol/l) or solvent alone. One hour later, $H_2O_2$ or solvent alone was added and cells were incubated for additional 24 h. Apoptosis was measured assessing the apoptotic fragmentation of cellular DNA. In cells transfected with cyclin D1 siRNA and incubated with both KN93 and $H_2O_2$, apoptosis was significantly decreased ($n=3$). In cells transfected with cyclin D1 siRNA, the difference between $H_2O_2$ and $H_2O_2$+KN93 treated cells is not significant. *Bottom)* Western blotting showing cyclin D1 down-modulation by siRNA.

## DISCUSSION

This study aimed at understanding the adaptive responses inducing enhanced EC survival in pro-oxidant conditions. Specifically, we found that oxidative stress induced rapid cyclin D1 down-modulation and that this event contributed to decrease EC death (**Fig. 10**).

In keeping with previous studies, in this study we used $H_2O_2$ concentrations within the submillimolar range. Although $H_2O_2$ concentrations measured in vivo are generally lower, one should consider the specificity of both the cell culture system and the $H_2O_2$ administration method. Indeed, we show that under the adopted conditions of cell density and growth factors presence, the apoptosis induced by 400 μmol/l $H_2O_2$ in asynchronous EC is negligible and 800 μmol/l $H_2O_2$ was insufficient to induce apoptosis in U2OS. To corroborate the relevance of our findings, we show that chemotherapics and ischemia, two red/ox imbalance inducing stimuli, both trigger cyclin D1 down-modulation.

Cyclin D1 down-modulation induced by $H_2O_2$ was due to its degradation via the ubiquitin-proteasome pathway. This finding is in agreement with previous studies indicating that cyclin D1 degradation is induced by several stress stimuli, including growth factor deprivation (15, 16), osmostic stress (17), UV (19, 39), and cisplatin damage (40).

Cyclin D1 degradation is regulated in specific circumstances by phosphorylation of Thr286 and Thr288 (15, 18, 20). We found that the expression of a specific cyclin D1 allele bearing mutations of both Thr286 and

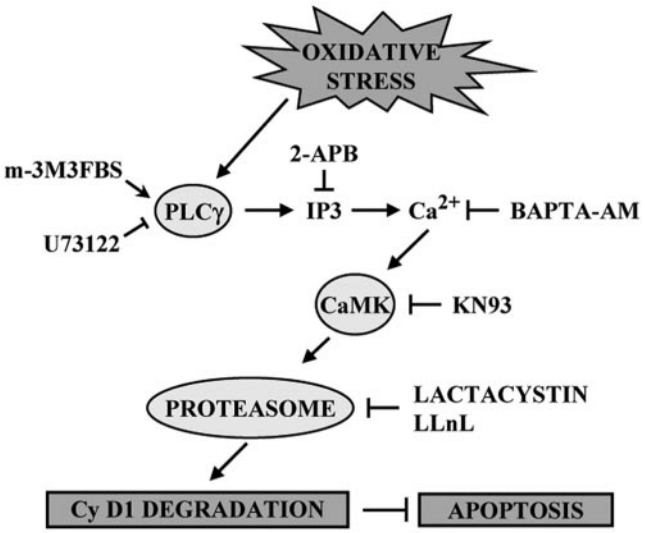


**Figure 10.** Diagram summarizing signal transduction pathway activated by $H_2O_2$ and leading to cyclin D1 degradation.

Thr288 was efficiently down-modulated by $H_2O_2$ treatment, suggesting that cyclin D1 phosphorylation by the kinases targeting these sites, namely GSK-3β, $p38^{Sapk2}$, and Mirk/dyrk1b, are not necessary for cyclin D1 demise induced by $H_2O_2$. In keeping with these data, cell treatment with LiCl, a potent inhibitor of GSK-3β, failed to prevent cyclin D1 down-modulation induced by $H_2O_2$ (L. Cicchillitti and F. Martelli, unpublished observations).

We also found that cyclin D1 turnover was regulated by PLC-dependent mobilization of intracellular $Ca^{2+}$. We did not formally prove that PLC isoforms different from PLC-γ were not involved in this event. However, to our knowledge, PLC-γ is the only PLC isoform described as activated by oxidative stress.

Both growth factors and ROS are capable of triggering PLC-γ activity and $Ca^{2+}$ mobilization, eliciting markedly different responses. Indeed, when quiescent cells are induced to proliferate by serum stimulation, cyclin D/CDK4 activity is stimulated by calmodulin (CaM) (41) and CaMK activities (42). Another relevant target on growth factors stimulation of quiescent cells is calcineurin, a $Ca^{2+}$/calmodulin-dependent phosphatase that positively regulates cyclin D1 translation (43). Modulation of responses to $[Ca^{2+}]_i$ increase can occur by coincident $Ca^{2+}$-independent signals, but there is also growing evidence that the strength, frequency, source, and location of the $Ca^{2+}$ signal are determinants for specific regulatory events. Athough further studies are needed to elucidate the differences between proliferative and oxidant stimuli, one distinguishing parameter is the duration of the stimulus: Unlike growth factors, $H_2O_2$ elicited a slowly developing and sustained $Ca^{2+}$ mobilization.

We also found that EC treatment with $H_2O_2$ induced CaMK activity and that this activity was necessary for cyclin D1 degradation. The most likely interpretation of our data is that $Ca^{2+}$ mobilization from internal stores leads to $Ca^{2+}$/calmodulin-dependent activation of CaMK. However, a $Ca^{2+}$/calmodulin-independent activation pathway of CaMK by $H_2O_2$ has been described, although in a different experimental system and on exposure to much higher concentrations of $H_2O_2$ (10 mmol/l) (44).

In a previous study, we showed that cell treatment with $H_2O_2$ induces a rapid arrest of S-phase progression that is dependent on the dephosphorylation of pRb by the PP2A phosphatase (21). A certain concentration of variability was observed between different batches of primary EC in the time course of both pRb dephosphorylation and cyclin D1 degradation; however, in all circumstances, cyclin D1 down-modulation always followed pRb dephosphorylation (21). Thus, it is unlikely that the main functional implication of $H_2O_2$-induced cyclin D1 degradation is pRb dephosphorylation. In keeping with this interpretation, we and others (45) found that re-expression of cyclin D1 in $H_2O_2$-treated cells was not sufficient to reactivate DNA synthesis.

Growing evidence indicates that cyclin D1 is involved in certain cases of neuronal apoptosis (11). High levels of cyclin D1 expression elicit an apoptotic response (12–14), indicating that increased cyclin D1 expression can present a survival challenge to the cell. We found that sublethal doses of $H_2O_2$ triggered significant apoptosis in cells over-expressing cyclin D1. Similarly, when cyclin D1 degradation was prevented by CaMK inhibition, cells were significantly sensitized to $H_2O_2$-induced apoptosis. Therefore, the reduction of endogenous cyclin D1 concentration observed after $H_2O_2$ treatment of EC must be mechanistically important in establishing cell survival. In agreement with these data, sensitivity to $H_2O_2$-induced cell death is increased in PLC-γ1 null cells, where cyclin D1 degradation in response to oxidants is likely disabled (6). Furthermore, cyclin D1 overexpression sensitizes cancer cells to fenretinide and adriamycin, two drugs inducing intracellular oxidative stress (46, 47). As expected, when cells were exposed to a lethal dose of $H_2O_2$, cell viability was not altered by the down modulation of cyclin D1 by RNAi (P. Fasanaro and F. Martelli, unpublished observations). These data indicate that cyclin D1 degradation has an antiapoptotic function that is physiologically relevant within the sublethal range of $H_2O_2$ dosage.

Both CDK-dependent and CDK-independent activities might contribute to cyclin D1 pro-apoptotic activity. Cyclin D1 expression may lead to unscheduled pRb phosphorylation and reactivation of certain E2F responsive genes that in turn may activate apoptosis (11). However, although CaMK inhibition prevented cyclin D1 degradation induced by $H_2O_2$, it failed to prevent pRb dephosphorylation (P. Fasanaro and F. Martelli, unpublished observations), suggesting that pRb may not be implicated in cyclin D1 sensitization to $H_2O_2$.

An alternative scenario is proposed by the evidence that cyclin D1 regulates DNA repair: cyclin D1 can bind

to PCNA inhibiting its functions (39, 48), and this may directly affect the ability of the cell to cope with DNA oxidative damage. In keeping with this observation, S-phase cells display an exquisitely high sensitivity to $H_2O_2$ on cyclin D1 re-expression.

Finally, cyclin D1 has been shown to affect the activity of various cellular transcription factors without the participation of CDKs and it is conceivable that the expression of apoptotic genes may be directly regulated by cyclin D1 (49). FJ

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## REFERENCES


1. Griendling, K. K., and FitzGerald, G. A. (2003) Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation* **108,** 2034–2040
2. Griendling, K. K., and FitzGerald, G. A. (2003) Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* **108,** 1912–1916
3. Martindale, J. L., and Holbrook, N. J. (2002) Cellular response to oxidative stress: signaling for suicide and survival. *J. Cell. Physiol.* **192,** 1–15
4. Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70,** 281–312
5. Qin, S., Inazu, T., and Yamamura, H. (1995) Activation and tyrosine phosphorylation of p72syk as well as calcium mobilization after hydrogen peroxide stimulation in peripheral blood lymphocytes. *Biochem. J.* **308,** 347–352
6. Wang, X. T., McCullough, K. D., Wang, X. J., Carpenter, G., and Holbrook, N. J. (2001) Oxidative stress-induced phospholipase C-gamma 1 activation enhances cell survival. *J. Biol. Chem.* **276,** 28364–28371
7. Newton, A. C. (2003) Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem. J.* **370,** 361–371
8. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev Mol. Cell. Biol.* **4,** 517–529
9. Hook, S. S., and Means, A. R. (2001) Ca(2+)/CaM-dependent kinases: from activation to function. *Annu. Rev. Pharmacol. Toxicol.* **41,** 471–505
10. Murray, A. W. (2004) Recycling the cell cycle: cyclins revisited. *Cell* **116,** 221–234
11. Liu, D. X., and Greene, L. A. (2001) Neuronal apoptosis at the G1/S cell cycle checkpoint. *Cell Tissue Res.* **305,** 217–228
12. Han, E. K., Begemann, M., Sgambato, A., Soh, J. W., Doki, Y., Xing, W. Q., Liu, W., and Weinstein, I. B. (1996) Increased expression of cyclin D1 in a murine mammary epithelial cell line induces p27kip1, inhibits growth, and enhances apoptosis. *Cell Growth Differ.* **7,** 699–710
13. Katayama, K., Dobashi, Y., Kitagawa, M., Kamekura, S., Kawai, M., Kadoya, Y., and Kameya, T. (2001) Overexpression of cdk4/cyclin D1 induces apoptosis in PC12 cells in the presence of trophic support. *FEBS Lett.* **509,** 382–388
14. Pratt, M. A., and Niu, M. Y. (2003) Bcl-2 controls caspase activation following a p53-dependent cyclin D1-induced death signal. *J. Biol. Chem.* **278,** 14219–14229
15. Diehl, J. A., Zindy, F., and Sherr, C. J. (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11,** 957–972
16. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12,** 3499–3511
17. Casanovas, O., Miro, F., Estanyol, J. M., Itarte, E., Agell, N., and Bachs, O. (2000) Osmotic stress regulates the stability of cyclin D1 in a p38SAPK2-dependent manner. *J. Biol. Chem.* **275,** 35091–35097
18. Zou, Y., Ewton, D. Z., Deng, X., Mercer, S. E., and Friedman, E. (2004) Mirk/dyrk1B kinase destabilizes cyclin D1 by phosphorylation at threonine 288. *J. Biol. Chem.* **279,** 27790–27798
19. Agami, R., and Bernards, R. (2000) Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **102,** 55–66
20. Germain, D., Russell, A., Thompson, A., and Hendley, J. (2000) Ubiquitination of free cyclin D1 is independent of phosphorylation on threonine 286. *J. Biol. Chem.* **275,** 12074–12079
21. Cicchillitti, L., Fasanaro, P., Biglioli, P., Capogrossi, M. C., and Martelli, F. (2003) Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *J. Biol. Chem.* **278,** 19509–19517
22. Martelli, F., Hamilton, T., Silver, D. P., Sharpless, N. E., Bardeesy, N., Rokas, M., DePinho, R. A., Livingston, D. M., and Grossman, S. R. (2001) p19ARF targets certain E2F species for degradation. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4455–4460
23. Zaccagnini, G., Martelli, F., Fasanaro, P., Magenta, A., Gaetano, C., Di Carlo, A., Biglioli, P., Giorgio, M., Martin-Padura, I., Pelicci, P. G., and Capogrossi, M. C. (2004) p66ShcA modulates tissue response to hindlimb ischemia. *Circulation* **109,** 2917–2923
24. Martelli, F., and Livingston, D. M. (1999) Regulation of endogenous E2F1 stability by the retinoblastoma family proteins. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2858–2863
25. Fucile, S., Palma, E., Mileo, A. M., Miledi, R., and Eusebi, F. (2000) Human neuronal threonine-for-leucine-248 alpha 7 mutant nicotinic acetylcholine receptors are highly Ca2+ permeable. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3643–3648
26. Browder, T., Butterfield, C. E., Kraling, B. M., Shi, B., Marshall, B., O'Reilly, M. S., and Folkman, J. (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res.* **60,** 1878–1886
27. Noda, T., Iwakiri, R., Fujimoto, K., and Aw, T. Y. (2001) Induction of mild intracellular redox imbalance inhibits proliferation of CaCo-2 cells. *FASEB J.* **15,** 2131–2139
28. Rutkowski, D. T., and Kaufman, R. J. (2004) A trip to the ER: coping with stress. *Trends Cell Biol.* **14,** 20–28
29. Bae, Y. S., Lee, T. G., Park, J. C., Hur, J. H., Kim, Y., Heo, K., Kwak, J. Y., Suh, P. G., and Ryu, S. H. (2003) Identification of a compound that directly stimulates phospholipase C activity. *Mol. Pharmacol.* **63,** 1043–1050
30. Hu, Q., Corda, S., Zweier, J. L., Capogrossi, M. C., and Ziegelstein, R. C. (1998) Hydrogen peroxide induces intracellular calcium oscillations in human aortic endothelial cells. *Circulation* **97,** 268–275
31. Cai, H., Davis, M. E., Drummond, G. R., and Harrison, D. G. (2001) Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1571–1576
32. Bilato, C., Curto, K. A., Monticone, R. E., Pauly, R. R., White, A. J., and Crow, M. T. (1997) The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the alphavbeta3 integrin complex is reversed by activated calcium/calmodulin- dependent protein kinase II. *J. Clin. Invest.* **100,** 693–704
33. Li, D. W., Liu, J. P., Mao, Y. W., Xiang, H., Wang, J., Ma, W. Y., Dong, Z., Pike, H. M., Brown, R. E., and Reed, J. C. (2005) Calcium-activated RAF/MEK/ERK signaling pathway mediates p53-dependent apoptosis and is abrogated by alphaB-crystallin through inhibition of RAS activation. *Mol. Biol. Cell* **16,** 4437–4453
34. Hardingham, G. E., Arnold, F. J., and Bading, H. (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. *Nat. Neurosci.* **4,** 565–566
35. Schmitt, J. M., Wayman, G. A., Nozaki, N., and Soderling, T. R. (2004) Calcium activation of ERK mediated by calmodulin kinase I. *J. Biol. Chem.* **279,** 24064–24072
36. Dolmetsch, R. E., Pajvani, U., Fife, K., Spotts, J. M., and Greenberg, M. E. (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science* **294,** 333–339

37. Chen, Q. M., Liu, J., and Merrett, J. B. (2000) Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. *Biochem. J.* **347,** 543–551
38. Leroy, C., Mann, C., and Marsolier, M. C. (2001) Silent repair accounts for cell cycle specificity in the signaling of oxidative DNA lesions. *EMBO J.* **20,** 2896–2906
39. Pagano, M., Theodoras, A. M., Tam, S. W., and Draetta, G. F. (1994) Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. *Genes Dev.* **8,** 1627–1639
40. Lan, Z., Sever-Chroneos, Z., Strobeck, M. W., Park, C. H., Baskaran, R., Edelmann, W., Leone, G., and Knudsen, E. S. (2002) DNA damage invokes mismatch repair-dependent cyclin D1 attenuation and retinoblastoma signaling pathways to inhibit CDK2. *J. Biol. Chem.* **277,** 8372–8381
41. Taules, M., Rius, E., Talaya, D., Lopez-Girona, A., Bachs, O., and Agell, N. (1998) Calmodulin is essential for cyclin-dependent kinase 4 (Cdk4) activity and nuclear accumulation of cyclin D1-Cdk4 during G1. *J. Biol. Chem.* **273,** 33279–33286
42. Kahl, C. R., and Means, A. R. (2004) Regulation of cyclin D1/Cdk4 complexes by calcium/calmodulin-dependent protein kinase I. *J. Biol. Chem.* **279,** 15411–15419
43. Kahl, C. R., and Means, A. R. (2004) Calcineurin regulates cyclin D1 accumulation in growth-stimulated fibroblasts. *Mol. Biol. Cell* **15,** 1833–1842
44. Howe, C. J., LaHair, M. M., McCubrey, J. A., and Franklin, R. A. (2004) Redox regulation of the CaM-kinases. *J. Biol. Chem.* **43,** 44573–44581.
45. Barnouin, K., Dubuisson, M. L., Child, E. S., Fernandez de Mattos, S., Glassford, J., Medema, R. H., Mann, D. J., and Lam, E. W. (2002) H2O2 induces a transient multi-phase cell cycle arrest in mouse fibroblasts through modulating cyclin D and p21Cip1 expression. *J. Biol. Chem.* **277,** 13761–13770
46. Pirkmaier, A., Yuen, K., Hendley, J., O'Connell, M. J., and Germain, D. (2003) Cyclin d1 overexpression sensitizes breast cancer cells to fenretinide. *Clin. Cancer Res.* **9,** 1877–1884
47. Shao, J., Teraishi, F., Katsuda, K., Tanaka, N., and Fujiwara, T. (2002) p53 inhibits adriamycin-induced down-regulation of cyclin D1 expression in human cancer cells. *Biochem. Biophys. Res. Commun.* **290,** 1101–1107
48. Fukami-Kobayashi, J., and Mitsui, Y. (1999) Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2. *Exp. Cell Res.* **246,** 338–347
49. Lamb, J., Ramaswamy, S., Ford, H. L., Contreras, B., Martinez, R. V., Kittrell, F. S., Zahnow, C. A., Patterson, N., Golub, T. R., and Ewen, M. E. (2003) A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* **114,** 323–334

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