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**MOL # 56473**

## Title Page

# REACTIVE OXYGEN SPECIES DECREASE CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN EXPRESSION IN CARDIOMYOCYTES VIA A PROTEIN KINASE D1-DEPENDENT MECHANISM THAT DOES NOT REQUIRE $Ser^{133}$ PHOSPHORYLATION

Nazira Özgen, Jianfen Guo, Zoya Gertsberg, Peter Danilo Jr., Michael R. Rosen, Susan F. Steinberg

*Center for Molecular Therapeutics (N.Ö., P.D. Jr., M.R.R., S. F. S.) and Departments of Pharmacology (N. Ö., J.G., Z.G., P.D. Jr., M.R.R., S. F. S.) and Pediatrics (N. Ö., M.R.R.), College of Physicians and Surgeons, Columbia University, New York, NY 10032*

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**RUNNING TITLE PAGE**

Running Title: ROS Decreases CREB Protein Expression via PKD1 in Cardiomyocytes

A) Correspondence:

Susan F. Steinberg, M.D.
Department of Pharmacology
College of Physicians and Surgeons
Columbia University
630 West 168 Street
New York, NY 10032
Telephone 212-305-4297
E-mail: sfs1@columbia.edu

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D) Abbreviations: β-gal, β-galactosidase; CRE, cyclic AMP response element; CREB, cAMP binding response element protein; GFX, GF109203X; HEK293 cells, human embryonic kidney cells; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MSK1, mitogen- and stress-activated protein kinase 1; PKA, protein kinase A; PKC, protein kinase C; PKD, protein kinase D; PSSA, phospho-site specific antibodies; ROS, reactive oxygen species; RNAi, RNA interference; RSK, p90 kDa ribosomal S6 kinase

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## Abstract

Reactive oxygen species (ROS) exert pleiotropic effects on a wide array of signaling proteins that regulate cellular growth and apoptosis. This study shows that chronic treatment with a low concentration of $H_2O_2$ leads to the activation of signaling pathways involving ERK, RSK, and PKD that increase cAMP binding response element protein (CREB) phosphorylation at $Ser^{133}$ in cardiomyocytes. While CREB-$Ser^{133}$ phosphorylation typically mediates cAMP-dependent increases in CREB target gene expression, the $H_2O_2$-dependent increase in CREB-$Ser^{133}$ phosphorylation is accompanied by a decrease in CREB protein abundance and no change in Cre-luciferase reporter activity. Mutagenesis studies indicate that $H_2O_2$ decreases CREB protein abundance via a mechanism that does not require CREB-$S^{133}$ phosphorylation. Rather, the $H_2O_2$-dependent decrease in CREB protein is prevented by the proteasome inhibitor lactacystin, by inhibitors of MEK or protein kinase C activity, or by adenoviral-mediated delivery of a small interfering RNA that decreases PKD1 expression. A PKD1-dependent mechanism that links oxidative stress to decreased CREB protein abundance is predicted to contribute to the pathogenesis of heart failure by influencing cardiac growth and apoptosis responses.

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## Introduction

CREB is a bZip transcription factor that binds to specific DNA elements (termed cAMP-response elements or CREs) within the regulatory regions of CREB target genes; many genes with CREs in their promoters play key roles in cellular proliferation and apoptosis pathways. CREB is regulated via phosphorylation at $Ser^{133}$, a modification variably attributed to protein kinase A (PKA), calcium/calmodulin-dependent kinase, p90 kDa ribosomal S6 kinase (RSK, an effector of the ERK-MAPK pathway), mitogen- and stress-activated protein kinase 1 (MSK1), protein kinase D (PKD) or AKT (depending upon the cell type or inciting stimulus; {Johannessen, 2004}). CREB-$Ser^{133}$ phosphorylation increases CREB-dependent gene transcription at least in part by recruiting coactivators (CREB binding protein or CBP) to the promoters of CREB target genes. In the heart, CREB is implicated in the maintenance of normal ventricular structure and function {Fentzke, 1998}. CREB protein expression decreases in a pacing-induced cardiac memory model in dogs that is characterized by a specialized form of electrical remodeling involving alterations in the T wave morphology during sinus rhythm {Patberg, 2003}. There is recent evidence that pacing protocols that induce cardiac memory lead to enhanced tissue markers of oxidative stress {Özgen, 2007}. It is tempting to speculate that this increase in ROS mediates the pacing-induced decrease CREB protein expression in cardiomyocytes, since oxidative stress decreases CREB protein abundance, depresses CRE-dependent gene transcription, and enhances apoptosis in neuronal and vascular smooth muscle cell models {Pugazhenthi, 2003}{Reusch, 2003}{Tokunou, 2003}. However, the ROS-activated pathways that regulate CREB protein abundance have not been examined. Moreover, while an ROS-dependent

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increase in CREB-Ser$^{133}$ phosphorylation is detected in many cellular models, the role of CREB-Ser$^{133}$ phosphorylation in the ROS-dependent decrease in CREB protein expression also has not been considered.

Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide, and hydroxy radicals contribute to the pathogenesis of ischemia-reperfusion injury, cardiac hypertrophy, heart failure, and other cardiovascular disorders associated with aging. ROS regulate the growth and survival of cardiomyocytes by modulating the activity of a wide array of signaling enzymes and transcription factors. However, cellular responses to oxidative stress can vary substantially, depending upon the magnitude and duration of the ROS stimulus {Kwon, 2003}. Low levels of ROS (typically associated with the activation of receptors that induce cardiac hypertrophy) act as second messengers to regulate signal transduction pathways that increase protein synthesis, promote cardiomyocyte growth, and/or induce ischemic preconditioning, typically without impairing cell survival. Higher levels of oxidative stress (that accompany cardiac ischemia and reperfusion injury) result in cell death, either through apoptosis and/or frank necrosis. While oxidative stress has been linked to the activation of an array of signaling enzymes with CREB-Ser$^{133}$ kinase activity, certain CRE-regulated genes (such as the cytoprotective Bcl-2 protein that inhibits the mitochondrial apoptosis pathway) are paradoxically *downregulated* by oxidative stress {Valks, 2003}{Pugazhenthi, 2003}. This study examines the ROS-regulated pathways that regulate CREB protein abundance and function in cardiomyocytes.

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## Material and Methods

***Cardiomyocyte culture and adenoviral infections*.** Cardiomyocytes were isolated from the hearts of 2-day-old Wistar rats by a trypsin dispersion procedure using a differential attachment procedure to enrich for cardiomyocytes followed by irradiation as detailed in previous publications{Steinberg, 1991}{Özgen, 2008}. The final yield of cells was typically 2.5-3 x $10^6$ per neonatal heart. Cells were plated on protamine sulfate-coated culture dishes at a density of $5x10^6$ cells/100-mm dish. Experiments were performed on cultures grown for 5 days in MEM (Gibco BRL) supplemented with 10% fetal calf serum and then serum-deprived for a subsequent 24 hr interval.

Cardiomyocytes were infected with adenoviral constructs that drive expression of β-galactosidase (β-gal), wild-type CREB (WT-CREB), non-phosphorylatable CREB-S133A (a CREB construct with a serine 133 to alanine mutation, generously provided by Dr. Jane Reusch), or an adenovirus that silences PKD1 expression (Ad-PKD1-RNAi, generously provided by Dr. Metin Avkiran); in each case, multiplicity of infection was 20 plaque forming units per cell.

***Western Blotting.*** Western blotting was performed on cell extracts according to methods described previously or manufacturer's instructions{Özgen, 2008}{Rybin, 2009}. The anti-PKD1-pSer$^{742}$ (numbering based upon human sequence, corresponding to Ser$^{748}$ in rodent PKD1) was from Abcam. All other antibodies were from Cell Signaling Technology. Studies that validate the specificity of the phospho-site specific antibodies (PSSAs) that recognize the phosphorylated forms of PKD1 are published

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{Rybin, 2009}{Jacamo, 2008}. Results in each figure represent data from a single experiment, at a single gel exposure, with all results replicated in at least 3 experiments on separate culture preparations.

***Luciferase assays.*** pCRE-luciferase (Strategene) and renilla luciferase (Promega) vectors were introduced into cardiomyocytes using a nucleofector kit (Amaxa Inc) according to manufactures' instruction. Cre-luciferase signals were normalized to luminescence from *Renilla* (to eliminate inter-assay variability and control for agonist-dependent changes in transfection efficiency or cell viability) using a dual-luciferase reporter assay system (Promega) according to methods described previously {Özgen, 2008}.

***Statistical analysis.*** All results are expressed as means $\pm$ S.E.M. Comparisons were made using one–way ANOVA or Student's t-test. $P<0.05$ was considered significant.

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## Results

*$H_2O_2$ decreases CREB protein content in cardiomyocytes*

Initial studies examined the effects of a range of $H_2O_2$ concentrations on signaling pathways that are predicted to regulate CREB-$Ser^{133}$ phosphorylation in cardiomyocytes. Stimulations were performed at 15 and 60 min, to discriminate transient responses from the more chronic changes in signal transduction pathways that are more likely to regulate gene expression and growth responses.

Fig 1A shows that $H_2O_2$ activates at least two enzymes with CREB-$Ser^{133}$ kinase activity. $H_2O_2$ induces a rapid increase in the phosphorylation of ERK and its downstream target RSK, a known CREB-$Ser^{133}$ kinase. All $H_2O_2$ concentrations increase ERK-RSK phosphorylation at the 15 min time point, although activation of the ERK-RSK cascade is most robust at 0.05-0.1 mM $H_2O_2$. The ERK-RSK pathway is activated by even lower $H_2O_2$ concentrations (0.01-0.05 mM) when the stimulation interval is prolonged to 60 min. $H_2O_2$ also activates PKD, a family of three structurally related serine/threonine kinases (PKD1, PKD2, and PKD3) that recently also have been implicated as CREB-$Ser^{133}$ kinases {Özgen, 2008}{Johannessen, 2007}. PKD activation is generally attributed to a phosphorylation-dependent mechanism involving protein kinase C (PKC). PKD activation is tracked by immunoblot analysis with PSSAs that specifically recognize the PKC-dependent phosphorylation of PKD at a pair of highly conserved serine residues in the activation loops of all three PKD isoforms (corresponding to $Ser^{744}$ and $Ser^{748}$ in rodent PKD1). PKD activation also is detected with a PSSA that recognizes an autophosphorylation at $Ser^{916}$, a site conserved at the

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extreme C-termini of PKD1 and PKD2, that is not conserved in PKD3; PKD1-Ser$^{916}$ autophosphorylation is widely used as a surrogate marker of PKD activation. Fig 1A shows that $H_2O_2$ increases PKD phosphorylation at both the activation loop (at Ser$^{744}$ and Ser$^{748}$) and at the C-terminal autophosphorylation site (at Ser$^{916}$). However, the effect of $H_2O_2$ to activate PKD is delayed, relative to $H_2O_2$-dependent ERK-RSK activation pathway. PKD activation is prominent in cardiomyocytes treated with 0.05-0.1 mM $H_2O_2$ for 60 min; little-to-no PKD activation is detected in cardiomyocytes treated with $H_2O_2$ for 15 min (or with $H_2O_2$ concentrations lower than 0.05 mM or higher than 0.1 mM for 60 min).

$H_2O_2$ also increases CREB-Ser$^{133}$ phosphorylation at both 15 and 60 min. The CREB-Ser$^{133}$ phosphorylation response at 15 min is greatest in cardiomyocytes treated with 0.05-0.1 mM $H_2O_2$; CREB-Ser$^{133}$ phosphorylation is less robust in cardiomyocytes treated for 15 min with either higher or lower $H_2O_2$ concentrations. This concentration-response relationship for $H_2O_2$-dependent CREB-Ser$^{133}$ phosphorylation parallels the concentration-response relationship for $H_2O_2$-dependent ERK-RSK activation. A 15 min treatment with $H_2O_2$ increases CREB-Ser$^{133}$ phosphorylation without changing CREB protein abundance.

$H_2O_2$ increases CREB-Ser$^{133}$ phosphorylation in association with a profound decrease in CREB protein abundance at 60 min. This is not due to a generalized decrease in protein recovery (and gross cytotoxicity), since the recovery of other proteins such as PKD and ERK is not impaired under these conditions. The antithetical effects of $H_2O_2$ on CREB protein abundance and CREB-Ser$^{133}$ phosphorylation also can not be attributed

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to an effect of $H_2O_2$ to increase the phosphorylation of other CREB/ATF transcription factor family members such as CREM and ATF-1 that in theory could be recognized by the anti-CREB-pSer$^{133}$ antibody. Fig 1B shows that CREM and ATF-1 are detected in neonatal rat cardiomyocytes, but their electrophoretic mobilities are considerably faster than the electrophoretic mobility of the CREB protein. CREM is detected as two isoforms, with mobilities corresponding to the CREM isoforms generated as a result of alternative splicing in uterine myometrium. $H_2O_2$ treatment leads to a decrease in abundance of the smaller CREMα isoform (MW 28-kDa, which has been implicated as a transcriptional repressor), but no change in the abundance of the larger CREMt2α isoform (MW 39-kDa, which has been implicated as a transcriptional activator). $H_2O_2$ treatment also leads to a decrease in the abundance of ATF-1. Additional studies showed that the anti-CREB-pSer$^{133}$ antibody recognizes a band that co-migrates with CREB, but it does not detect bands that co-migrate with CREM or ATF-1, in $H_2O_2$-treated cardiomyocytes. Effects of $H_2O_2$ on CREB, CREMτ2α, CREMα and ATF-1 protein abundance, and CREB-Ser$^{133}$ phosphorylation, are quantified in Fig 1C.

Fig 1A shows that treatment with 0.1 mM $H_2O_2$ for 60 min leads to an increase in CREB-Ser$^{133}$ phosphorylation in association with a marked increase in PKD phosphorylation, and only a low level of ERK-RSK activation. Higher $H_2O_2$ concentrations (0.5-1 mM) increase CREB-Ser$^{133}$ phosphorylation without increasing ERK-RSK or PKD phosphorylation, presumably through a mechanism that involves the activation of a different CREB-Ser$^{133}$ kinase or the inhibition of a CREB-Ser$^{133}$ phosphatase.

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Fig 2 provides a more detailed analysis of $H_2O_2$ regulation of CREB, showing that 0.05 mM $H_2O_2$ induces a transient increase in CREB-Ser$^{133}$ phosphorylation that is confined to the initial 60 min of stimulation; CREB-Ser$^{133}$ phosphorylation wanes when the $H_2O_2$ stimulation interval is prolonged to 2-24 hr. In contrast, treatment with 0.05 mM $H_2O_2$ leads to a decrease CREB protein abundance that is sustain for up to 72 hrs. Control experiments show that forskolin (an adenylyl cyclase activator that increases PKA activity) increases CREB-Ser$^{133}$ phosphorylation without decreasing CREB protein content. These results indicate that long-lasting changes in CREB-Ser$^{133}$ phosphorylation do not necessarily lead to decreased CREB protein abundance in cardiomyocytes.

While CREB-Ser$^{133}$ phosphorylation is sufficient to induce a CRE-dependent transcription response in cells treated with agonists that activate the cAMP-PKA pathway, the CREB transcriptional activity in cardiomyocytes treated with $H_2O_2$ is difficult to predict since [1] CREB-Ser$^{133}$ phosphorylation is generally not sufficient to induce transcription in response to agonist that activate other growth regulatory pathways and [2] the stimulatory effect of increased CREB-Ser$^{133}$ phosphorylation might be functionally offset by the decrease in CREB protein abundance. Therefore, effects of $H_2O_2$ on CRE-luciferase assay were directly examined. Fig 2B shows that treatment with 0.05 mM $H_2O_2$ does not lead to a detectable increase in CRE-luciferase activity (at any time point from 1-24 hrs). Control experiments show that a 6 hr stimulation with forskolin leads to a robust increase in CRE-luciferase activity. These results indicate that the $H_2O_2$-dependent increase in CREB-Ser$^{133}$ phosphorylation is not associated with a transcriptional response.

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*The $H_2O_2$-dependent signaling pathways that regulate CREB protein; the role of $Ser^{133}$ phosphorylation.*

The kinetic studies suggest that $H_2O_2$ might promote CREB-$Ser^{133}$ phosphorylation via different signaling pathway at very short (15-30 min) and more protracted (60 min) stimulation intervals, since [1] $H_2O_2$ increases CREB-$Ser^{133}$ phosphorylation in association with a pronounced increase in ERK-RSK activity (and little-to-no increase in PKD activity) at 15-30 min, whereas [2] 0.1 mM $H_2O_2$ increases CREB-$Ser^{133}$ phosphorylation in association with a pronounced increase in PKD, and only a low level of ERK-RSK activation, at 60 min. We used pharmacologic inhibitors as an initial strategy to examine the role of ERK-RSK and PKC-PKD pathways in $H_2O_2$-dependent mechanisms that regulate CREB.

Fig 3 shows that GF109203X (an inhibitor of PKC isoforms that also displays some inhibitory activity toward RSK) abrogates $H_2O_2$-dependent ERK-RSK and PKD activation and CREB-$Ser^{133}$ phosphorylation at both 30 and 60 min; GF109203X also prevents the $H_2O_2$-dependent decrease in CREB protein content. In contrast, U0126 (an inhibitor of MEK, the upstream ERK kinase) blocks the rapid $H_2O_2$-dependent increase in ERK, RSK, and CREB-$Ser^{133}$ phosphorylation at 30 min and the late $H_2O_2$-dependent fall in CREB protein content at 60 min; U0126 does not interfere with the $H_2O_2$-dependent pathway that activates PKD and it does not prevent the late $H_2O_2$-dependent increase in CREB-$Ser^{133}$ phosphorylation at 60 min. The observation that U0126 prevents the $H_2O_2$-dependent decrease in CREB protein content, without fully blocking CREB-$Ser^{133}$ phosphorylation, suggests that $H_2O_2$ decreases CREB protein content via a mechanism that does not require $Ser^{133}$ phosphorylation. Of note, while $H_2O_2$ treatment

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leads to the activation of many other signaling pathways (Guo et al., 2009;Clerk et al., 1998), inhibitors that abrogate the $H_2O_2$-dependent increases in p38-MAPK, JNK, or AKT activity did not prevent the $H_2O_2$-dependent fall in CREB protein expression (data not shown).

On the basis of previous studies showing that CREB is polyubiquitinated and targeted to the proteosome for degradation in pancreatic β-cells treated with high glucose {Costes, 2009}, and that CREB is targeted for proteasomal degradation via a phosphorylation-dependent mechanism in hypoxic epithelial cells {Taylor, 2000}, we examined whether the $H_2O_2$ dependent decreases in CREB protein abundance can be blocked by lactacystin (an inhibitor of protein degradation by the proteosome). Fig 4 shows that lactacystin prevents the $H_2O_2$-dependent decrease in CREB protein abundance, suggesting a role for the proteasomal degradation pathway.

We then examined the role of CREB-$Ser^{133}$ phosphorylation in the $H_2O_2$-dependent mechanism that regulates CREB protein abundance using a mutagenesis strategy. Cardiomyocytes were infected with adenoviruses that drive overexpression of WT- and S133A-substituted forms of CREB and then treated with vehicle or 0.05 mM $H_2O_2$ for 60 min. Figure 5 shows that the effect of $H_2O_2$ to regulate CREB is retained in this overexpression model. $H_2O_2$ treatment leads to a similar increase in $Ser^{133}$ phosphorylation, and a decreased in CREB protein abundance, for both the endogenous CREB protein (in β-gal infected cultures) and the heterologously overexpressed WT-CREB transgene. The CREB mutant harboring a S133A substitution (that is not regulated by $H_2O_2$ through $Ser^{133}$ phosphorylation) also is downregulated by the $H_2O_2$

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treatment protocol, indicating that $H_2O_2$ downregulates CREB protein content via a proteosomal mechanism that does not require $Ser^{133}$ phosphorylation.

*$H_2O_2$ downregulates CREB via a PKD1-dependent mechanism*

The observation that GF109203X prevents the $H_2O_2$-dependent decrease in CREB protein abundance suggests that CREB protein levels are regulated by PKC and/or a PKC-activated enzyme such as PKD. Since the specificity of most available pharmacologic inhibitors is imperfect, we used a gene silencing approach to examine the role of PKD1 in the $H_2O_2$-dependent mechanism that regulates CREB protein expression in cardiomyocytes. Fig 6 shows that an adenoviral vector designed to silence PKD1 expression (Ad-PKD1-RNAi) specifically decreases PKD1 expression (without any associated changes in PKD2 expression) and that PKD1 downregulation also prevents the $H_2O_2$-dependent decrease in CREB protein expression (at both 60 min and 72 hr). Collectively, these experiments implicate PKD1 as a $H_2O_2$-activated enzyme that regulates CREB protein abundance via a mechanism that does not require CREB-$Ser^{133}$ phosphorylation.

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## Discussion

The major findings of this study are that [1] low physiologic concentrations of $H_2O_2$ activate the ERK-RSK and PKD pathways, promote CREB-Ser$^{133}$ phosphorylation and decrease CREB protein content in cultured neonatal rat cardiomyocytes, [2] the $H_2O_2$-dependent increase in CREB-S$^{133}$ phosphorylation is not sufficient to induce a CRE-dependent transcriptional response, and [3] $H_2O_2$ decreases CREB protein content via a mechanism that requires PKD1, but does not require CREB phosphorylation at Ser$^{133}$.

While CREB-Ser$^{133}$ phosphorylation leads to increased CREB-mediated transcription in cardiomyocytes treated with forskolin (an agonist that increases cAMP), the $H_2O_2$-dependent pathway leading to CREB-Ser$^{133}$ phosphorylation does not increase CRE-luciferase activity. Similar discrepancies between CREB-Ser$^{133}$ phosphorylation and CRE-dependent gene transcription have been identified in studies of other agonists that act via cAMP-PKA-independent pathways. A high level of CREB-Ser$^{133}$ phosphorylation that is not associated with a CRE-dependent transcriptional response has been attributed to inhibitory modifications elsewhere on CREB that destabilize the CREB-CBP complex {Wagner, 2000 #212}{Mayr, 2001 #213} and/or the failure to recruit additional signal-regulated coactivators that cooperate with CBP/p300 to regulate CREB target gene expression{Ravnskjaer, 2007 #214}. The ROS-dependent decrease in CREB protein abundance identified in this study might constitute a third mechanism that would conspire to limit CREB target gene expression.

These studies provide novel evidence that $H_2O_2$ decreases CREB protein abundance via a mechanism involving PKD1. The initial focus on PKD as a potential

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mediator of ROS-dependent effects on CREB was based upon recent studies implicating PKD as a CREB-$S^{133}$ kinase. While studies with pharmacologic inhibitors suggest that PKD contributes to the $H_2O_2$-dependent CREB-$S^{133}$ phosphorylation response under certain conditions, studies with the CREB-S133A mutant indicate that $H_2O_2$ regulates CREB protein levels via a PKD-dependent pathway that does not require $S^{133}$ phosphorylation. Other mechanisms must be considered. In theory, PKD might regulate CREB protein levels by phosphorylating CREB at a site other than $Ser^{133}$. In fact, Johannessen et al. identified $Ser^{98}$ (which resides in a PKD consensus phosphorylation motif) as an alternative site on CREB that functions *in vitro* as a phosphoaccepter site for PKD {Johannessen, 2007 #170}. While these investigators did not detect PKD-$Ser^{98}$ phosphorylation in epidermoid carcinoma cells treated with PKD-activating stimuli or in HEK293 cells engineered to ectopically-overexpress an activated form of the PKD1 enzyme, an $H_2O_2$-dependent pathway involving PKD (or a PKD-activated effector) that regulates CREB phosphorylation at a site other than $Ser^{133}$ in cardiomyocytes remains possible and is the focus of ongoing studies. Alternatively, the $H_2O_2$-dependent decrease in CREB protein abundance might be due to the phosphorylation of a different cellular PKD substrate that regulates CREB protein levels indirectly.

The observation that $H_2O_2$ decreases CREB protein abundance via a mechanism that is specific for PKD1 is quite significant. In general, studies that have used RNAi or genetic knockout strategies to downregulate PKD1 have exposed a high level of functional redundancy between PKD1 and other PKD isoforms that are co-expressed in most cells. For example, agonist-dependent pathways involving PKD that promote class II histone deacetylase phosphorylation in lymphocytes and cardiomyocytes are inhibited

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only when PKD1 and other PKD isoforms are silenced simultaneously {Matthews, 2006 #172}. The loss of PKD1 alone also does not prevent agonist-dependent CREB-$Ser^{133}$ phosphorylation responses in epidermoid carcinoma cells{Johannessen, 2007 #169}. Preliminary studies show that the PKD1 downregulation protocol (that abrogates the $H_2O_2$-dependent decrease in CREB protein abundance) also does not prevent CREB-$Ser^{133}$ phosphorylation in cardiomyocytes (data not shown), providing further evidence that $Ser^{133}$ phosphorylation is not required for $H_2O_2$-dependent regulation of CREB protein abundance.

While the gene silencing studies implicate PKD1 in the $H_2O_2$-dependent pathway that regulates CREB protein content, pharmacologic studies with U0126 suggest that the MEK-ERK pathway also regulates this process. While MEK-ERK and PKD1 pathways might act independently, PKD1 could regulate CREB protein abundance indirectly by controlling signaling via the ERK cascade, since PKD1 can phosphorylate RIN, a Ras binding protein that prevents Ras-Raf interactions; RIN dissociates from Ras when it is phosphorylated by PKD, leading to enhanced signaling via the Raf-ERK pathway {Wang, 2002 #173}.

Decreased CREB protein expression is implicated in the pathogenesis of certain rodent models of oxidative stress-induced vascular injury and atherosclerosis. This study implicates PKD1 in a similar ROS-regulated pathway that decreases CREB expression and disrupts CREB-mediated transcription in cardiomyocytes. This mechanism may contribute to the acquisition of cardiac memory, a specialized form of electrical remodeling that follows a period of altered ventricular activation and is characterized by increased tissue markers of oxidative stress, decreased CREB protein abundance,

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decreased expression of KChIP2 (an accessory subunit of the transient outward potassium current $I_{to}$ that contains a cAMP response element in its promoter) and altered T wave morphology (Patberg et al., 2005). An oxidative stress-dependent mechanism involving PKD1 that disrupts CREB responses also may contribute to the pathogenesis of age-related diseases such as diabetes, lipid disorders that are characterized by increased oxidative stress.

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## References

Clerk A, Fuller S J, Michael A and Sugden P H (1998) Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J Biol Chem* 273:7228-7234.

Fentzke RC, Korcarz C E, Lang R M, Lin H and Leiden J M (1998) Dilated cardiomyopathy in transgenic mice expressing a dominant-negative CREB transcription factor in the heart. *J Clin Invest* 101:2415-2426.

Guo J, Gertsberg Z, Özgen N and Steinberg S F (2009) p66Shc links $\alpha_1$-adrenergic receptors to a reactive oxygen species-dependent AKT-FOXO3a phosphorylation pathway in cardiomyocytes. *Circ Res.*

Jacamo R, Sinnett-Smith J, Rey O, Waldron R T and Rozengurt E (2008) Sequential PKC-dependent and PKC-independent protein kinase D catalytic activation via Gq-coupled receptors: Differential regulation of activation loop $Ser^{744}$ and $Ser^{748}$ phosphorylation. *J Biol Chem* 283:12877-12887.

Johannessen M, Delghandi M P and Moens U (2004) What turns CREB on? *Cell Signal* 16:1211-1227.

Johannessen M, Delghandi M P, Rykx A, Dragset M, Vandenheede J R, Van Lint J and Moens U (2007) Protein kinase D induces transcription through direct phosphorylation of the cAMP-response element-binding protein. *J Biol Chem* 282:14777-14787.

Kwon SH, Pimentel D R, Remondino A, Sawyer D B and Colucci W S (2003) $H_2O_2$ regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways. *J Mol Cell Cardiol* 35:615-621.

Mayr BM, Canettieri G and Montminy M R (2001) Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB. *Proc Natl Acad Sci U S A* 98:10936-10941.

Özgen N, Obreztchikova M, Guo J, Elouardighi H, Dorn G W, Wilson B A and Steinberg S F (2008) Protein kinase D links Gq-coupled receptors to cAMP response element-binding protein (CREB)-$Ser^{133}$ phosphorylation in the heart. *J Biol Chem* 283:17009-17019.

Özgen, N., Plotnikov, A. N., Shlapakova, I., Dankort, D., Steinberg, S. F., and Rosen, M. R. A reactive oxygen species-mediated PKC-ERK-RSK pathway decreases the cyclic AMP response element binding protein and may initiate cardiac memory. Circulation 116, II-88. 2007.

Patberg KW, Obreztchikova M N, Giardina S F, Symes A J, Plotnikov A N, Qu J, Chandra P, McKinnon D, Liou S R, Rybin A V, Shlapakova I, Danilo P, Jr., Yang J and Rosen M R (2005) The cAMP response element binding protein modulates expression of the transient outward current: implications for cardiac memory. *Cardiovasc Res* 68:259-267.

Pugazhenthi S, Nesterova A, Jambal P, Audesirk G, Kern M, Cabell L, Eves E, Rosner M R, Boxer L M and Reusch J E (2003) Oxidative stress-mediated down-regulation of Bcl-2 promoter in hippocampal neurons. *J Neurochem* 84:982-996.

Ravnskjaer K, Kester H, Liu Y, Zhang X, Lee D, Yates J R, III and Montminy M (2007) Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. *EMBO J* 26:2880-2889.

Reusch JE and Klemm D J (2003) Cyclic AMP response element-binding protein in the vessel wall: good or bad? *Circulation* 108:1164-1166.

Rybin VO, Guo J and Steinberg S F (2009) Protein kinase D1 autophosphorylation via distinct mechanisms at $Ser^{744}/Ser^{748}$ and $Ser^{916}$. *J Biol Chem* 284:2332-2343.

Steinberg SF, Robinson R B, Lieberman H B, Stern D M and Rosen M R (1991) Thrombin modulates phosphoinositide metabolism, cytosolic calcium, and impulse initiation in the heart. *Circ Res* 68:1216-1229.

Tokunou T, Shibata R, Kai H, Ichiki T, Morisaki T, Fukuyama K, Ono H, Iino N, Masuda S, Shimokawa H, Egashira K, Imaizumi T and Takeshita A (2003) Apoptosis induced by inhibition of cyclic AMP response element-binding protein in vascular smooth muscle cells. *Circulation* 108:1246-1252.

Valks DM, Kemp T J and Clerk A (2003) Regulation of Bcl-XL expression by $H_2O_2$ in cardiac myocytes. *J Biol Chem* 278:25542-25547.

Wagner BL, Bauer A, Schutz G and Montminy M (2000) Stimulus-specific interaction between activator-coactivator cognates revealed with a novel complex-specific antiserum. *J Biol Chem* 275:8263-8266.

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## Footnotes

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## Legends for Figures

**FIGURE 1. $H_2O_2$-ACTIVATED SIGNALING PATHWAYS THAT REGULATE CREB PROTEIN ABUNDANCE AND CREB-SER$^{133}$ PHOSPHORYLATION IN NEONATAL RAT CARDIAC MYOCYTES.** Cell extracts from cardiomyocytes treated with the indicated concentrations of $H_2O_2$ for 15 min or 60 min were subjected to Western blotting with the indicated antibodies. Representative data are depicted in *Panels A* and *B* with the results for CREB-$S^{133}$ phosphorylation and CREB, CREMτ2α, CREMα, and ATF-1 protein content following a 60 min treatment with 0.05 mM $H_2O_2$ quantified in *Panel C.* In panel B, the molecular weight markers are applied to all Westerns. *, $p<0.05$ versus Control (n=5).

**FIGURE 2. $H_2O_2$ INCREASES CREB-SER$^{133}$ PHOSPHORYLATION WITHOUT INCREASING CRE-LUCIFERASE ACTIVITY IN CARDIOMYOCYTTES.** *Panel A*: Cardiomyocytes were treated with vehicle, $H_2O_2$ (50μM) or forskolin (10 μM) for indicated time intervals and cell extracts were subjected to Western blotting with indicated antibodies. *Panel B*: The CRE-firefly luciferase reporter construct and a *Renilla* luciferase vector (included as an internal control to normalized for differences in transfection efficiency) were introduced into cardiomyocytes by electroporation. Cells were treated with vehicle, $H_2O_2$ (0.05 mM) or forskolin (Fors, 10μM, as a positive control) for indicated intervals and CRE-firefly luciferase activity was corrected for minor differences in *Renilla* luciferase activity (n=4). * $p<0.05$ versus control.

**FIGURE 3. THE ROLE OF ERK-RSK AND PKD IN $H_2O_2$-DEPENDENT REGULATION OF CREB.** Immunoblotting on cell extracts from cardiomyocytes pretreated for 45 min with

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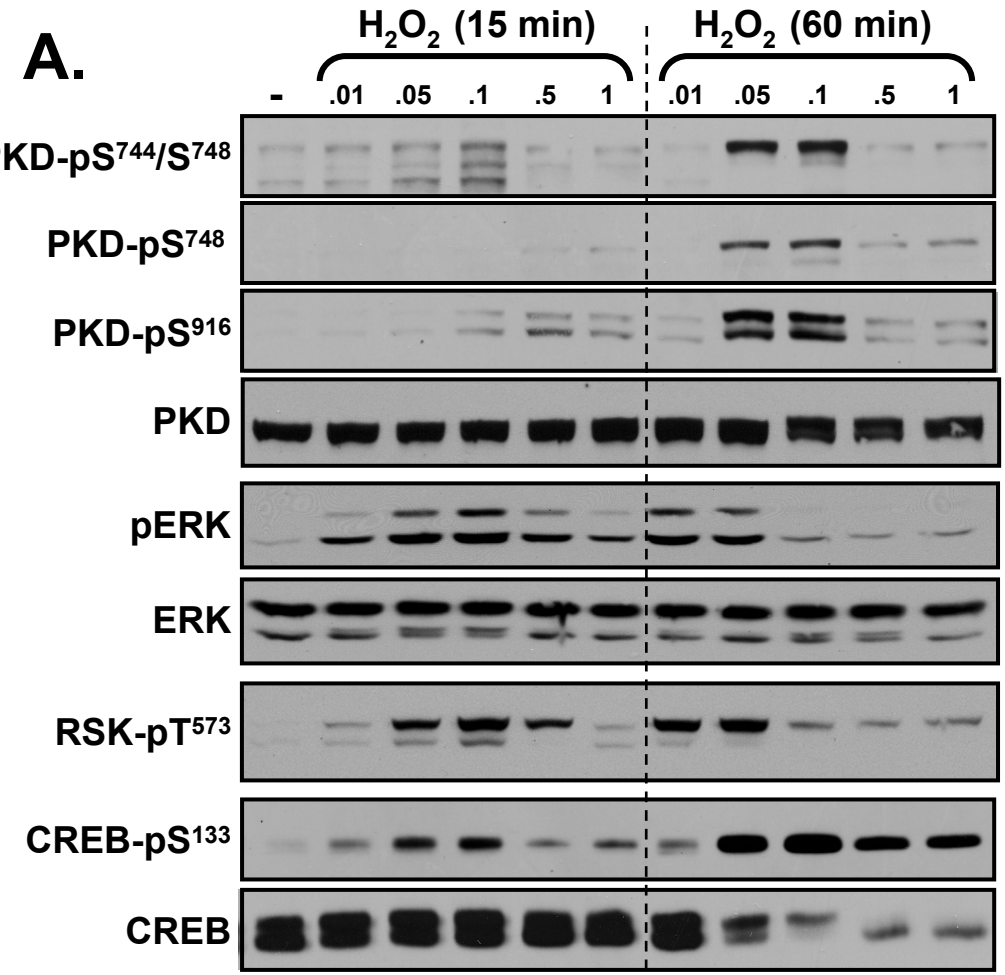
vehicle, GF109203X (GFX, 10 μM) or U0126 (5 μM) and then challenged with vehicle or $H_2O_2$ (0.05 mM) for the indicated intervals. Representative results are depicted on the top, with the results quantified at the bottom. *, $p<0.05$ vs control (n=3).

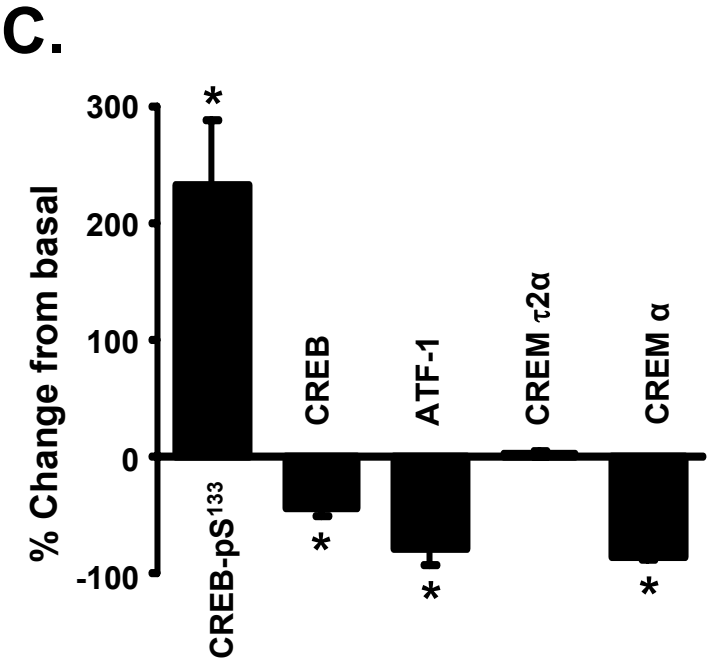
**FIGURE 4. LACTACYSTIN PREVENTS THE $H_2O_2$-DEPENDENT DECREASE IN CREB PROTEIN CONTENT.** Extracts from cultures pretreated for 30 min with lactacystin (10uM) and then challenged for 1 hr with vehicle or $H_2O_2$ (0.05 mM) were subjected to Western blotting for CREB phosphorylation and CREB protein content. Similar results were obtained in 2 other experiments.

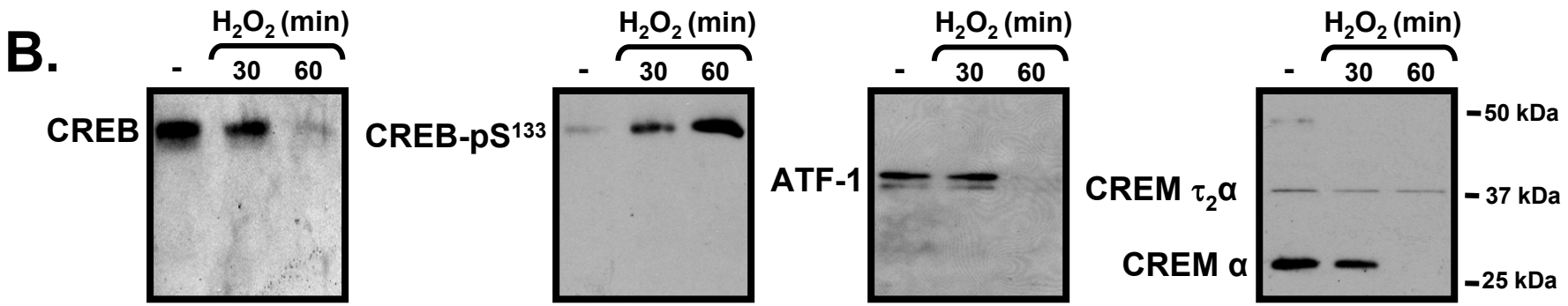
**FIGURE 5. $H_2O_2$ DECREASES CREB PROTEIN CONTENT THROUGH A MECHANISM THAT DOES NOT REQUIRE CREB-SER$^{133}$ PHOSPHORYLATION.** Adenoviral-mediated gene transfer was used to overexpress β-gal, WT-CREB, or CREB-S133A. Cultures were treated with vehicle or $H_2O_2$ (0.05 mM) for 1 hr and cell extracts were subjected to Western blotting for CREB phosphorylation and CREB protein content. Similar results were obtained in 2 other experiments.

**FIGURE 6. PKD1 KNOCKDOWN PREVENTS THE $H_2O_2$-DEPENDENT DECREASE IN CREB PROTEIN CONTENT.** Cardiomyocyte cultures were infected with the Ad-PKD1-RNAi or Ad-β-gal as a control. Cells were treated with vehicle or 0.05 mM $H_2O_2$ for 1 hr (Panel A) or 72 hrs (Panel B) (starting 3 days following adenoviral infections). Cell extracts were subjected to immunoblot analysis for PKD1 and CREB protein content. Data were replicated in 3 separate experiments on separate culture preparations.

Figure 1


A.
H2O2 (15 min)
H2O2 (60 min)
- .01 .05 .1 .5 1 .01 .05 .1 .5 1
PKD-pS744/S748
PKD-pS748
PKD-pS916
PKD
pERK
ERK
RSK-pT573
CREB-pS133
CREB


C.
% Change from basal
300
200
100
0
-100
CREB-pS133
CREB
ATF-1
CREM τ2α
CREM α
*


B.
H2O2 (min)
- 30 60
CREB
CREB-pS133
ATF-1
CREM τ2α
CREM α
50 kDa
37 kDa
25 kDa

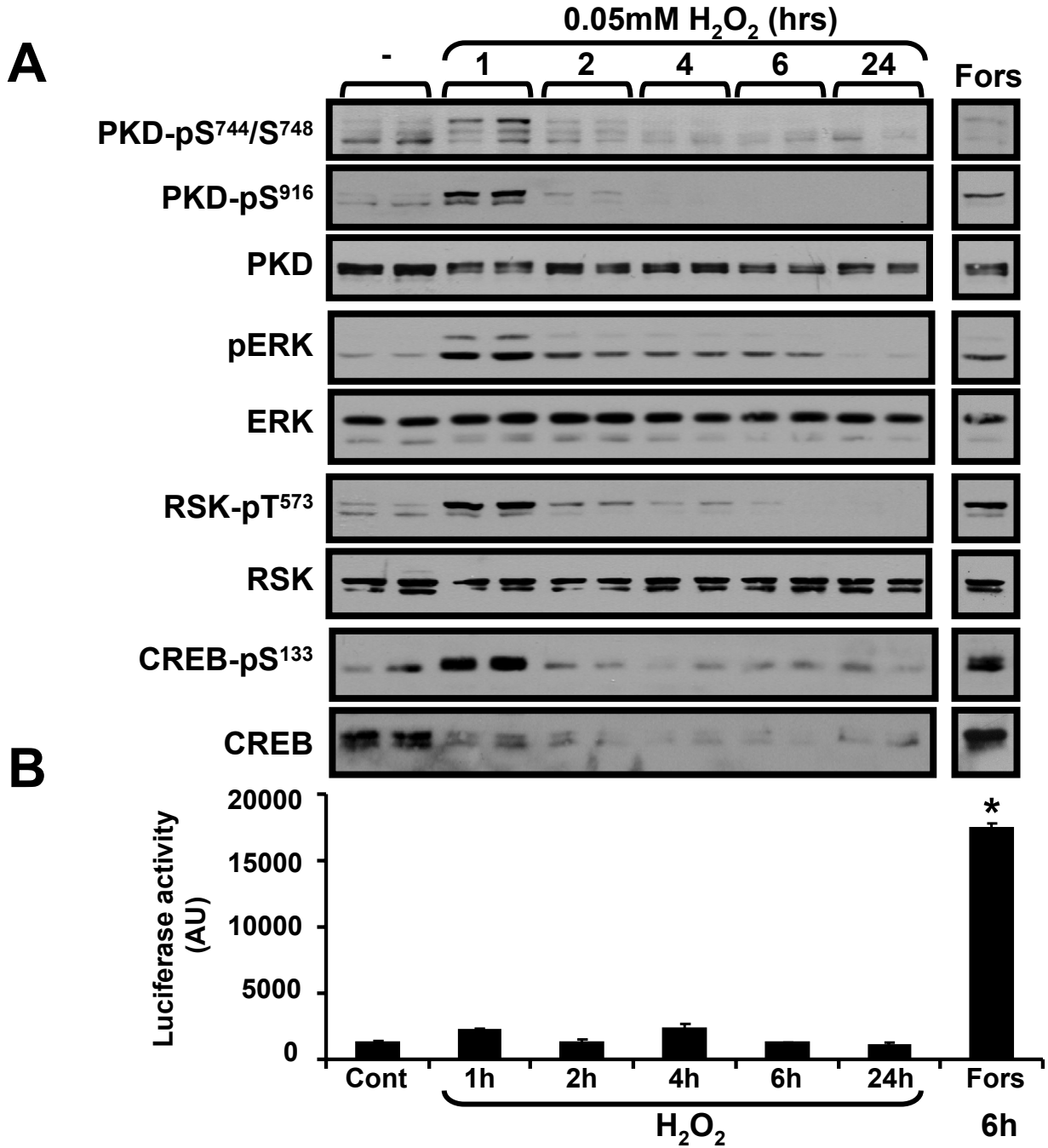
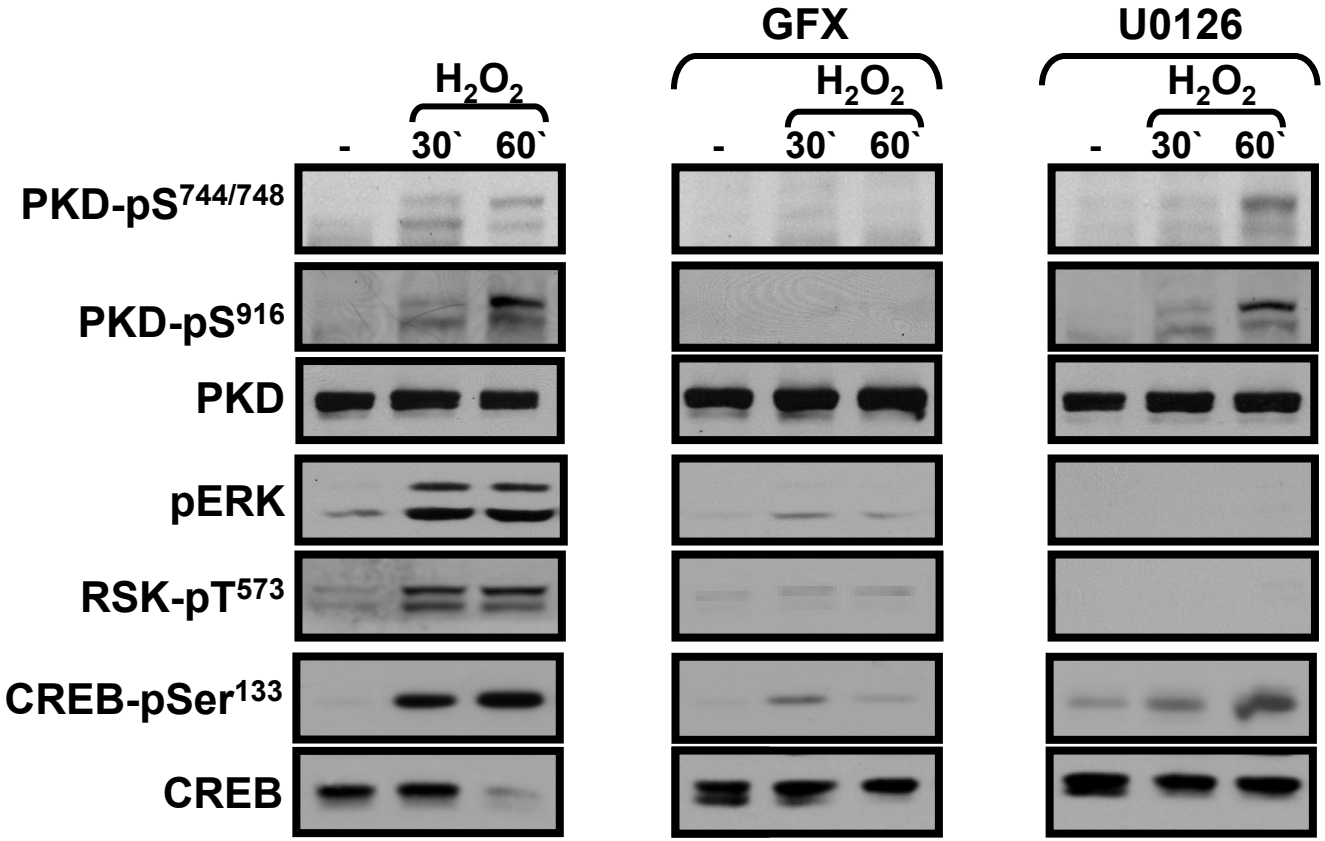

A
0.05mM $H_2O_2$ (hrs)
-
1
2
4
6
24
Fors
PKD-pS744/S748
PKD-pS916
PKD
pERK
ERK
RSK-pT573
RSK
CREB-pS133
CREB
B
Luciferase activity (AU)
20000
15000
10000
5000
0
*
Cont
1h
2h
4h
6h
24h
Fors
$H_2O_2$
6h

Figure 2


GFX
U0126
$H_2O_2$
-
30`
60`
PKD-pS744/748
PKD-pS916
PKD
pERK
RSK-pT573
CREB-pSer133
CREB

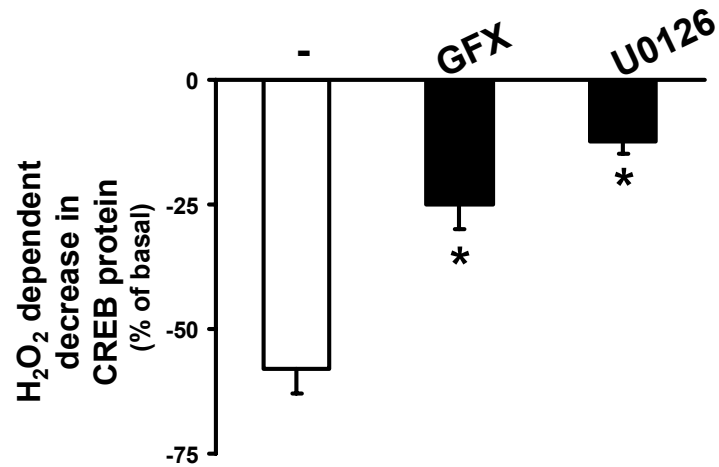

-
GFX
U0126
0
-25
-50
-75
$H_2O_2$ dependent decrease in CREB protein (% of basal)
*
*

Figure 3

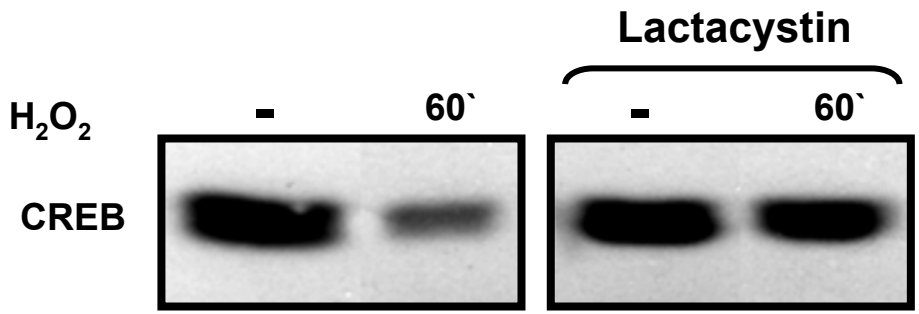



Figure 4

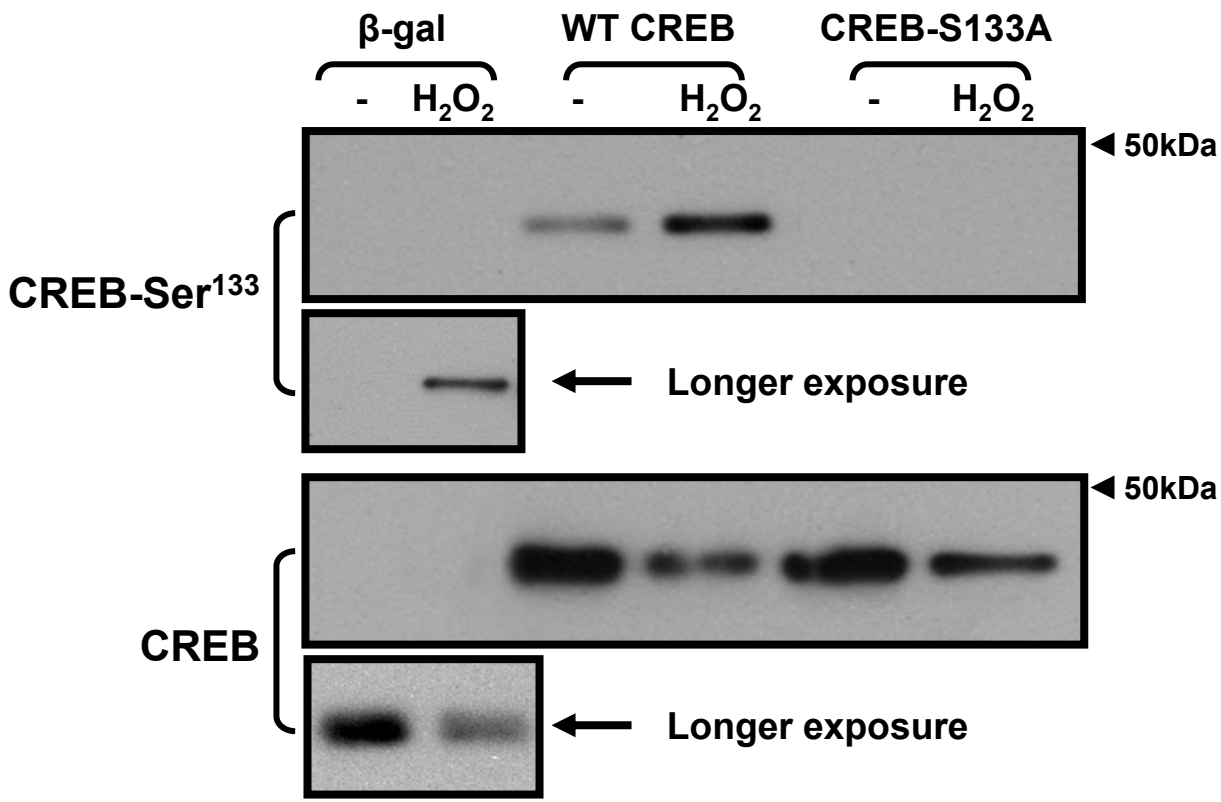

β-gal
WT CREB
CREB-S133A
-
$H_2O_2$
-
$H_2O_2$
-
$H_2O_2$
50kDa
CREB-Ser133
Longer exposure
50kDa
CREB
Longer exposure

Figure 5

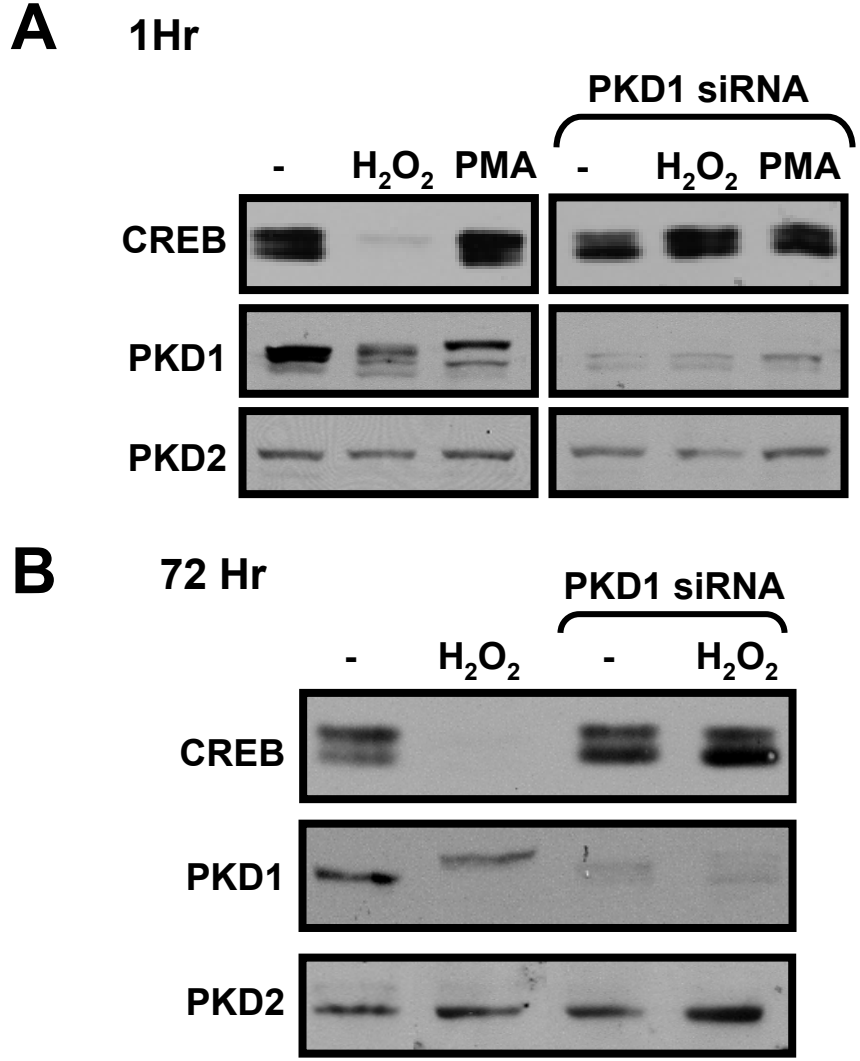

A
1Hr
PKD1 siRNA
-
$H_2O_2$
PMA
-
$H_2O_2$
PMA
CREB
PKD1
PKD2
B
72 Hr
PKD1 siRNA
-
$H_2O_2$
-
$H_2O_2$
CREB
PKD1
PKD2

Figure 6