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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 Ser¹³³ 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: 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 H₂O₂ leads to the activation of signaling pathways involving ERK, RSK, and PKD that increase cAMP binding response element protein (CREB) phosphorylation at Ser¹³³ in cardiomyocytes. While CREB-Ser¹³³ phosphorylation typically mediates cAMP-dependent increases in CREB target gene expression, the H₂O₂-dependent increase in CREB-Ser¹³³ phosphorylation is accompanied by a decrease in CREB protein abundance and no change in Cre-luciferase reporter activity. Mutagenesis studies indicate that H₂O₂ decreases CREB protein abundance via a mechanism that does not require CREB-S¹³³ phosphorylation. Rather, the H₂O₂-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 Ser¹³³, 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-Ser¹³³ 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 {Pugazhenthii, 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-Ser¹³³ phosphorylation is detected in many cellular models, the role of CREB-Ser¹³³ phosphorylation in the ROS-dependent decrease in CREB protein expression also has not been considered.

Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), 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-Ser¹³³ 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}{Pugazhenti, 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 $2.5\text{-}3 \times 10^6$ per neonatal heart. Cells were plated on protamine sulfate-coated culture dishes at a density of 5×10^6 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-pSer⁷⁴² (numbering based upon human sequence, corresponding to Ser⁷⁴⁸ 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 (Stratagene) 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

H₂O₂ decreases CREB protein content in cardiomyocytes

Initial studies examined the effects of a range of H₂O₂ concentrations on signaling pathways that are predicted to regulate CREB-Ser¹³³ 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 H₂O₂ activates at least two enzymes with CREB-Ser¹³³ kinase activity. H₂O₂ induces a rapid increase in the phosphorylation of ERK and its downstream target RSK, a known CREB-Ser¹³³ kinase. All H₂O₂ 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 H₂O₂. The ERK-RSK pathway is activated by even lower H₂O₂ concentrations (0.01-0.05 mM) when the stimulation interval is prolonged to 60 min. H₂O₂ also activates PKD, a family of three structurally related serine/threonine kinases (PKD1, PKD2, and PKD3) that recently also have been implicated as CREB-Ser¹³³ 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 Ser⁷⁴⁴ and Ser⁷⁴⁸ in rodent PKD1). PKD activation also is detected with a PSSA that recognizes an autophosphorylation at Ser⁹¹⁶, a site conserved at the

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extreme C-termini of PKD1 and PKD2, that is not conserved in PKD3; PKD1-Ser⁹¹⁶ autophosphorylation is widely used as a surrogate marker of PKD activation. Fig 1A shows that H₂O₂ increases PKD phosphorylation at both the activation loop (at Ser⁷⁴⁴ and Ser⁷⁴⁸) and at the C-terminal autophosphorylation site (at Ser⁹¹⁶). However, the effect of H₂O₂ to activate PKD is delayed, relative to H₂O₂-dependent ERK-RSK activation pathway. PKD activation is prominent in cardiomyocytes treated with 0.05-0.1 mM H₂O₂ for 60 min; little-to-no PKD activation is detected in cardiomyocytes treated with H₂O₂ for 15 min (or with H₂O₂ concentrations lower than 0.05 mM or higher than 0.1 mM for 60 min).

H₂O₂ also increases CREB-Ser¹³³ phosphorylation at both 15 and 60 min. The CREB-Ser¹³³ phosphorylation response at 15 min is greatest in cardiomyocytes treated with 0.05-0.1 mM H₂O₂; CREB-Ser¹³³ phosphorylation is less robust in cardiomyocytes treated for 15 min with either higher or lower H₂O₂ concentrations. This concentration-response relationship for H₂O₂-dependent CREB-Ser¹³³ phosphorylation parallels the concentration-response relationship for H₂O₂-dependent ERK-RSK activation. A 15 min treatment with H₂O₂ increases CREB-Ser¹³³ phosphorylation without changing CREB protein abundance.

H₂O₂ increases CREB-Ser¹³³ 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 H₂O₂ on CREB protein abundance and CREB-Ser¹³³ phosphorylation also can not be attributed

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to an effect of H₂O₂ 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-pSer¹³³ 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. H₂O₂ treatment leads to a decrease in abundance of the smaller CREM α isoform (MW 28-kDa, which has been implicated as a transcriptional repressor), but no change in the abundance of the larger CREM τ 2 α isoform (MW 39-kDa, which has been implicated as a transcriptional activator). H₂O₂ treatment also leads to a decrease in the abundance of ATF-1. Additional studies showed that the anti-CREB-pSer¹³³ antibody recognizes a band that co-migrates with CREB, but it does not detect bands that co-migrate with CREM or ATF-1, in H₂O₂-treated cardiomyocytes. Effects of H₂O₂ on CREB, CREM τ 2 α , CREM α and ATF-1 protein abundance, and CREB-Ser¹³³ phosphorylation, are quantified in Fig 1C.

Fig 1A shows that treatment with 0.1 mM H₂O₂ for 60 min leads to an increase in CREB-Ser¹³³ phosphorylation in association with a marked increase in PKD phosphorylation, and only a low level of ERK-RSK activation. Higher H₂O₂ concentrations (0.5-1 mM) increase CREB-Ser¹³³ phosphorylation without increasing ERK-RSK or PKD phosphorylation, presumably through a mechanism that involves the activation of a different CREB-Ser¹³³ kinase or the inhibition of a CREB-Ser¹³³ phosphatase.

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Fig 2 provides a more detailed analysis of H₂O₂ regulation of CREB, showing that 0.05 mM H₂O₂ induces a transient increase in CREB-Ser¹³³ phosphorylation that is confined to the initial 60 min of stimulation; CREB-Ser¹³³ phosphorylation wanes when the H₂O₂ stimulation interval is prolonged to 2-24 hr. In contrast, treatment with 0.05 mM H₂O₂ 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-Ser¹³³ phosphorylation without decreasing CREB protein content. These results indicate that long-lasting changes in CREB-Ser¹³³ phosphorylation do not necessarily lead to decreased CREB protein abundance in cardiomyocytes.

While CREB-Ser¹³³ 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 H₂O₂ is difficult to predict since [1] CREB-Ser¹³³ 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-Ser¹³³ phosphorylation might be functionally offset by the decrease in CREB protein abundance. Therefore, effects of H₂O₂ on CRE-luciferase assay were directly examined. Fig 2B shows that treatment with 0.05 mM H₂O₂ 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 H₂O₂-dependent increase in CREB-Ser¹³³ phosphorylation is not associated with a transcriptional response.

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The H₂O₂-dependent signaling pathways that regulate CREB protein; the role of Ser¹³³ phosphorylation.

The kinetic studies suggest that H₂O₂ might promote CREB-Ser¹³³ phosphorylation via different signaling pathway at very short (15-30 min) and more protracted (60 min) stimulation intervals, since [1] H₂O₂ increases CREB-Ser¹³³ 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 H₂O₂ increases CREB-Ser¹³³ 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 H₂O₂-dependent mechanisms that regulate CREB.

Fig 3 shows that GF109203X (an inhibitor of PKC isoforms that also displays some inhibitory activity toward RSK) abrogates H₂O₂-dependent ERK-RSK and PKD activation and CREB-Ser¹³³ phosphorylation at both 30 and 60 min; GF109203X also prevents the H₂O₂-dependent decrease in CREB protein content. In contrast, U0126 (an inhibitor of MEK, the upstream ERK kinase) blocks the rapid H₂O₂-dependent increase in ERK, RSK, and CREB-Ser¹³³ phosphorylation at 30 min and the late H₂O₂-dependent fall in CREB protein content at 60 min; U0126 does not interfere with the H₂O₂-dependent pathway that activates PKD and it does not prevent the late H₂O₂-dependent increase in CREB-Ser¹³³ phosphorylation at 60 min. The observation that U0126 prevents the H₂O₂-dependent decrease in CREB protein content, without fully blocking CREB-Ser¹³³ phosphorylation, suggests that H₂O₂ decreases CREB protein content via a mechanism that does not require Ser¹³³ phosphorylation. Of note, while H₂O₂ 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 H₂O₂-dependent increases in p38-MAPK, JNK, or AKT activity did not prevent the H₂O₂-dependent fall in CREB protein expression (data not shown).

On the basis of previous studies showing that CREB is polyubiquitinated and targeted to the proteasome 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 H₂O₂ dependent decreases in CREB protein abundance can be blocked by lactacystin (an inhibitor of protein degradation by the proteasome). Fig 4 shows that lactacystin prevents the H₂O₂-dependent decrease in CREB protein abundance, suggesting a role for the proteasomal degradation pathway.

We then examined the role of CREB-Ser¹³³ phosphorylation in the H₂O₂-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 H₂O₂ for 60 min. Figure 5 shows that the effect of H₂O₂ to regulate CREB is retained in this overexpression model. H₂O₂ treatment leads to a similar increase in Ser¹³³ 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 H₂O₂ through Ser¹³³ phosphorylation) also is downregulated by the H₂O₂

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treatment protocol, indicating that H₂O₂ downregulates CREB protein content via a proteosomal mechanism that does not require Ser¹³³ phosphorylation.

H₂O₂ downregulates CREB via a PKD1-dependent mechanism

The observation that GF109203X prevents the H₂O₂-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 H₂O₂-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 H₂O₂-dependent decrease in CREB protein expression (at both 60 min and 72 hr). Collectively, these experiments implicate PKD1 as a H₂O₂-activated enzyme that regulates CREB protein abundance via a mechanism that does not require CREB-Ser¹³³ phosphorylation.

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Discussion

The major findings of this study are that [1] low physiologic concentrations of H₂O₂ activate the ERK-RSK and PKD pathways, promote CREB-Ser¹³³ phosphorylation and decrease CREB protein content in cultured neonatal rat cardiomyocytes, [2] the H₂O₂-dependent increase in CREB-S¹³³ phosphorylation is not sufficient to induce a CRE-dependent transcriptional response, and [3] H₂O₂ decreases CREB protein content via a mechanism that requires PKD1, but does not require CREB phosphorylation at Ser¹³³.

While CREB-Ser¹³³ phosphorylation leads to increased CREB-mediated transcription in cardiomyocytes treated with forskolin (an agonist that increases cAMP), the H₂O₂-dependent pathway leading to CREB-Ser¹³³ phosphorylation does not increase CRE-luciferase activity. Similar discrepancies between CREB-Ser¹³³ 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-Ser¹³³ 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 H₂O₂ 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-S¹³³ kinase. While studies with pharmacologic inhibitors suggest that PKD contributes to the H₂O₂-dependent CREB-S¹³³ phosphorylation response under certain conditions, studies with the CREB-S133A mutant indicate that H₂O₂ regulates CREB protein levels via a PKD-dependent pathway that does not require S¹³³ phosphorylation. Other mechanisms must be considered. In theory, PKD might regulate CREB protein levels by phosphorylating CREB at a site other than Ser¹³³. In fact, Johannessen et al. identified Ser⁹⁸ (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-Ser⁹⁸ 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 H₂O₂-dependent pathway involving PKD (or a PKD-activated effector) that regulates CREB phosphorylation at a site other than Ser¹³³ in cardiomyocytes remains possible and is the focus of ongoing studies. Alternatively, the H₂O₂-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 H₂O₂ 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-Ser¹³³ phosphorylation responses in epidermoid carcinoma cells {Johannessen, 2007 #169}. Preliminary studies show that the PKD1 downregulation protocol (that abrogates the H₂O₂-dependent decrease in CREB protein abundance) also does not prevent CREB-Ser¹³³ phosphorylation in cardiomyocytes (data not shown), providing further evidence that Ser¹³³ phosphorylation is not required for H₂O₂-dependent regulation of CREB protein abundance.

While the gene silencing studies implicate PKD1 in the H₂O₂-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 α_1 -adrenergic receptors to a reactive oxygen species-dependent AKT-FOXO3a phosphorylation pathway in cardiomyocytes. *Circ Res*.

Jacamo R, Sinnott-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 Ser⁷⁴⁴ and Ser⁷⁴⁸ 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) H₂O₂ 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, Obreztkhikova 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)-Ser¹³³ 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.

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Patberg KW, Obreztkhikova 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.

Pugazhenth 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 Ser⁷⁴⁴/Ser⁷⁴⁸ and Ser⁹¹⁶. *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 H₂O₂ 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. H₂O₂-ACTIVATED SIGNALING PATHWAYS THAT REGULATE CREB PROTEIN ABUNDANCE AND CREB-SER¹³³ PHOSPHORYLATION IN NEONATAL RAT CARDIAC MYOCYTES. Cell extracts from cardiomyocytes treated with the indicated concentrations of H₂O₂ 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-S¹³³ phosphorylation and CREB, CREM τ 2 α , CREM α , and ATF-1 protein content following a 60 min treatment with 0.05 mM H₂O₂ 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. H₂O₂ INCREASES CREB-SER¹³³ PHOSPHORYLATION WITHOUT INCREASING CRE-LUCIFERASE ACTIVITY IN CARDIOMYOCYTTES. *Panel A:* Cardiomyocytes were treated with vehicle, H₂O₂ (50 μ M) or forskolin (10 μ M) 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, H₂O₂ (0.05 mM) or forskolin (Fors, 10 μ M, 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 H₂O₂-DEPENDENT REGULATION OF CREB. Immunoblotting on cell extracts from cardiomyocytes pretreated for 45 min with

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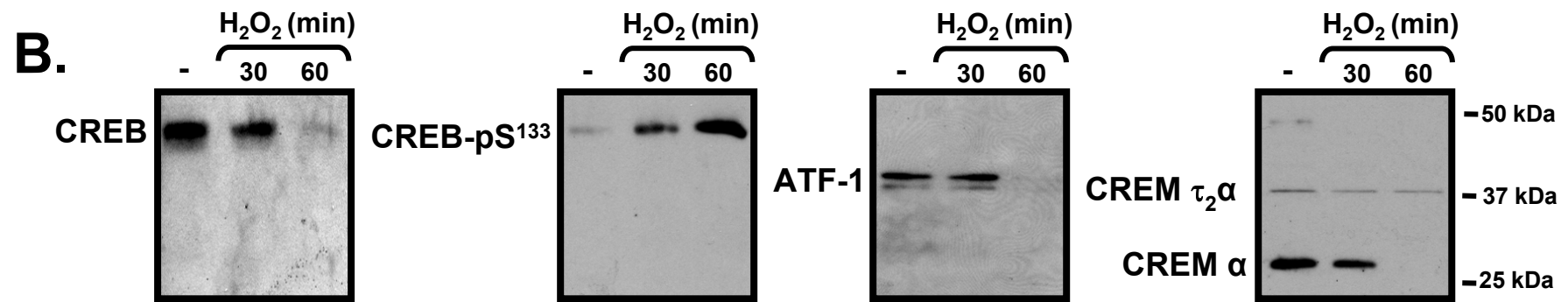
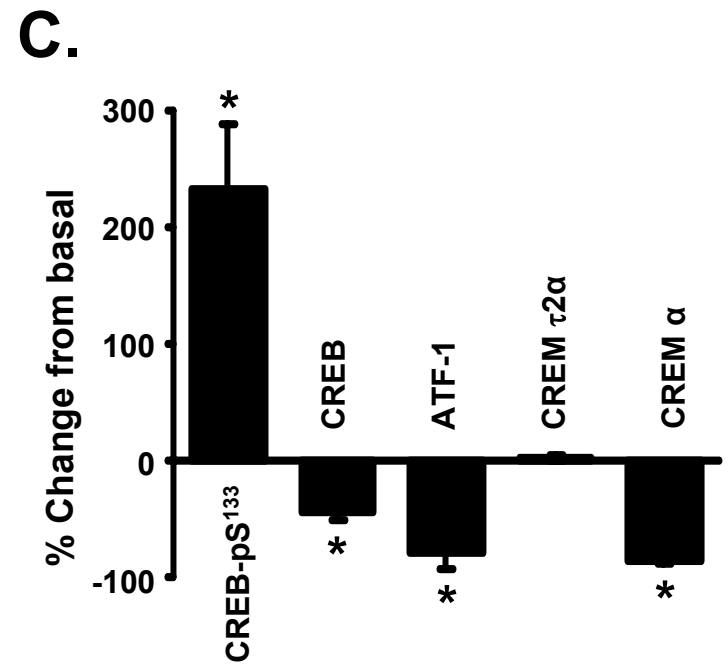
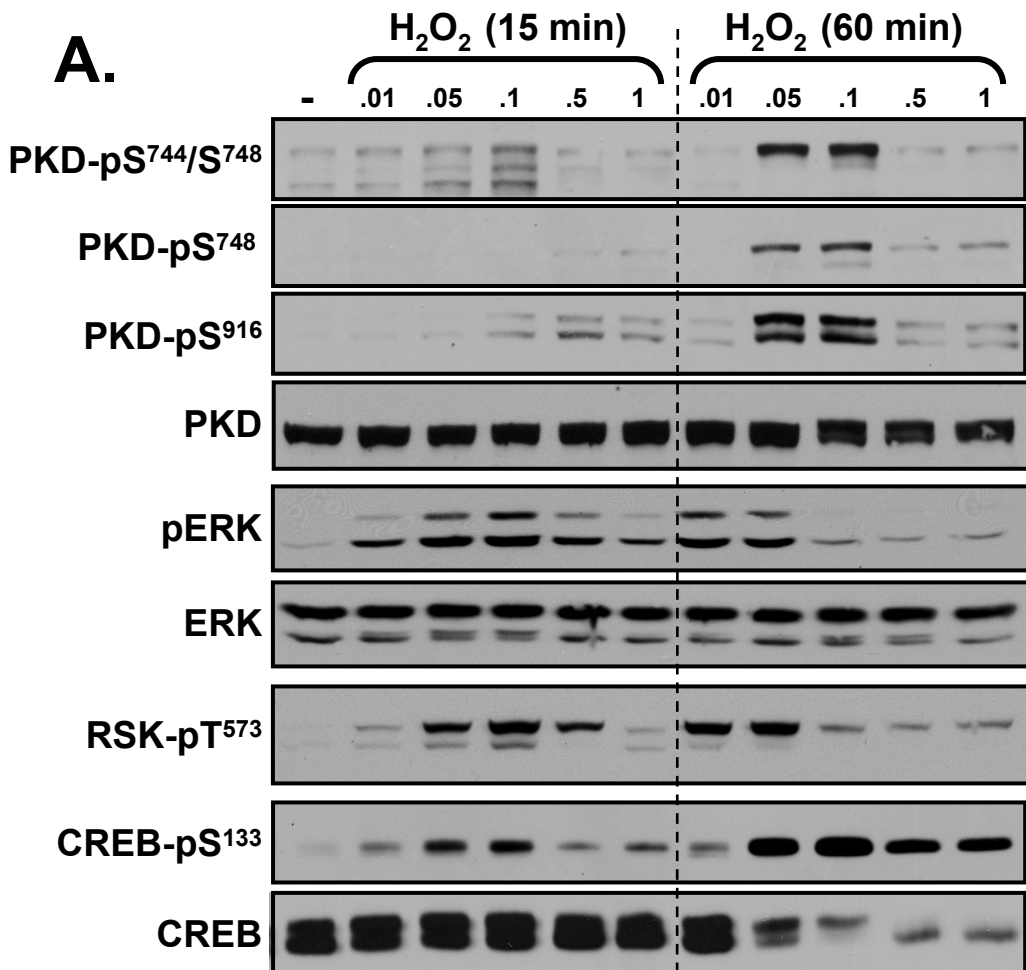
vehicle, GF109203X (GFX, 10 μ M) or U0126 (5 μ M) and then challenged with vehicle or H₂O₂ (0.05 mM) for the indicated intervals. Representative results are depicted on the top, with the results quantified at the bottom. *, p<0.05 vs control (n=3).

FIGURE 4. LACTACYSTIN PREVENTS THE H₂O₂-DEPENDENT DECREASE IN CREB PROTEIN CONTENT. Extracts from cultures pretreated for 30 min with lactacystin (10 μ M) and then challenged for 1 hr with vehicle or H₂O₂ (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. H₂O₂ DECREASES CREB PROTEIN CONTENT THROUGH A MECHANISM THAT DOES NOT REQUIRE CREB-SER¹³³ PHOSPHORYLATION. Adenoviral-mediated gene transfer was used to overexpress β -gal, WT-CREB, or CREB-S133A. Cultures were treated with vehicle or H₂O₂ (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 H₂O₂-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 H₂O₂ 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



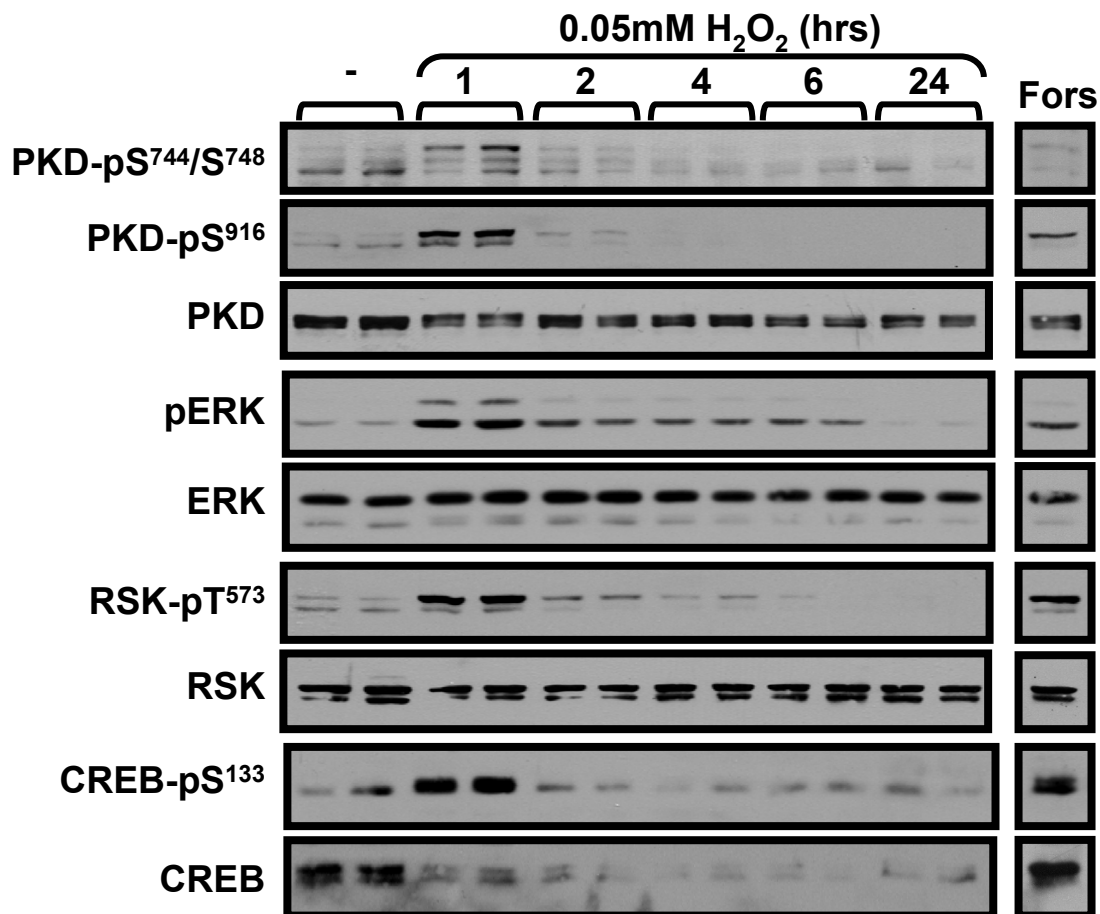
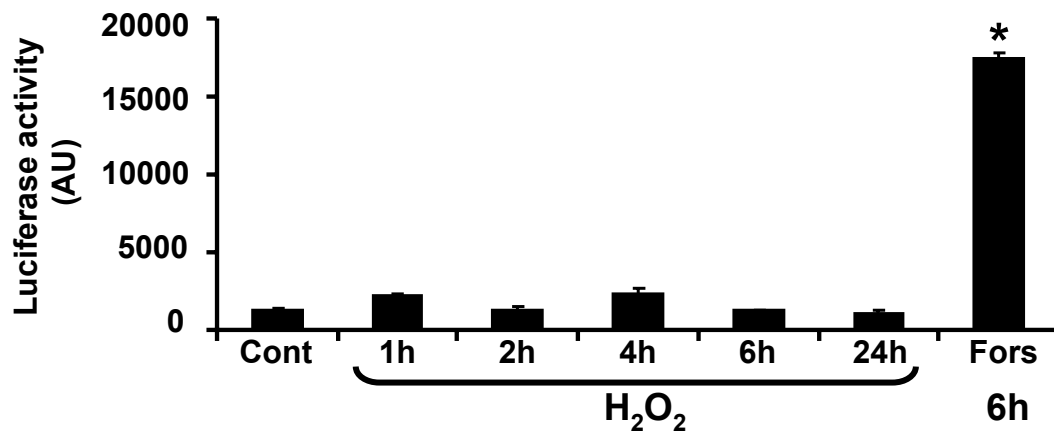
A**B**

Figure 2

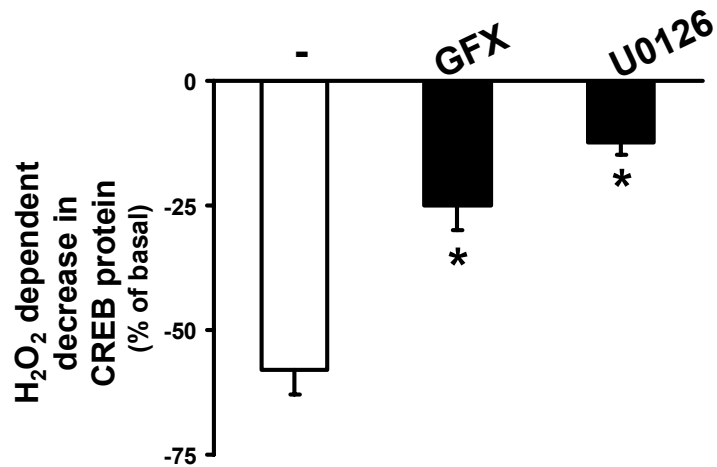
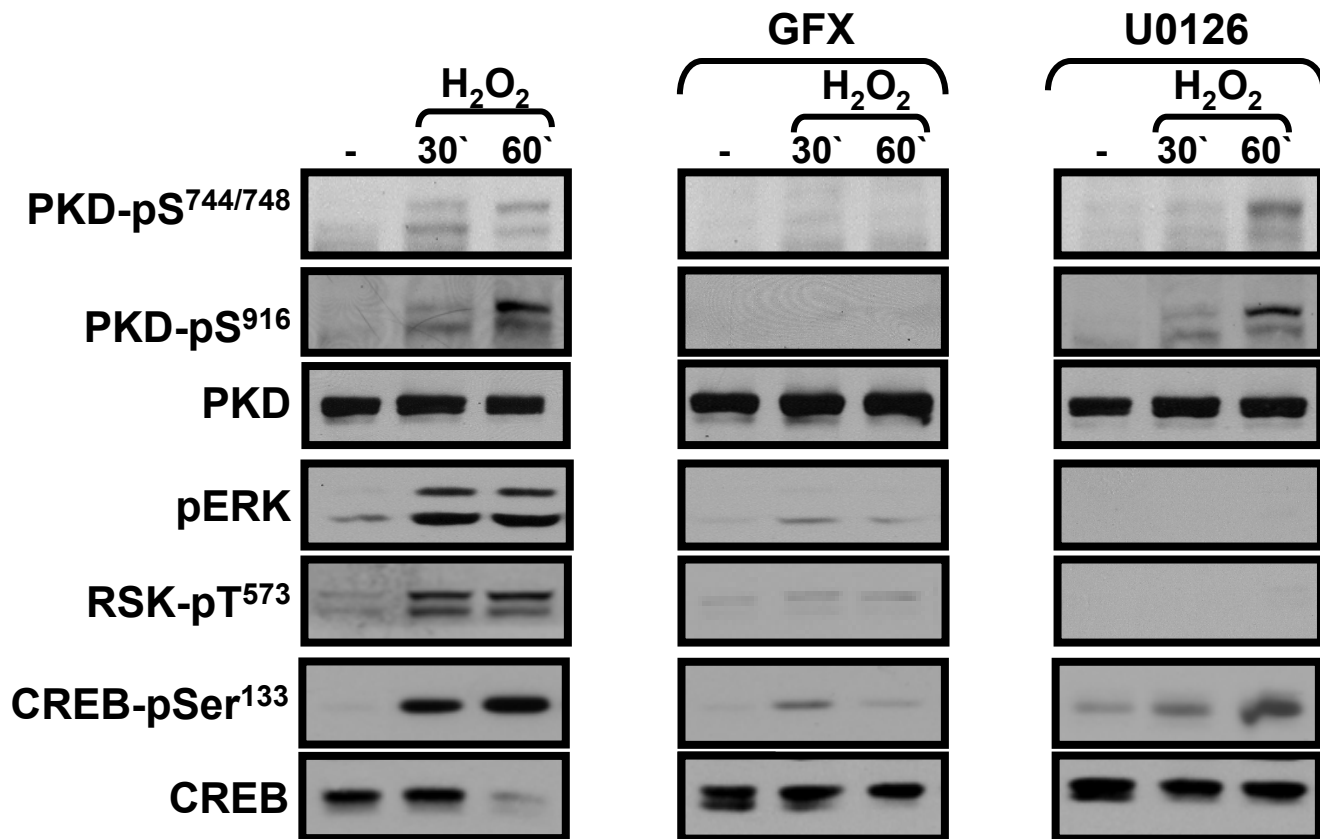


Figure 3

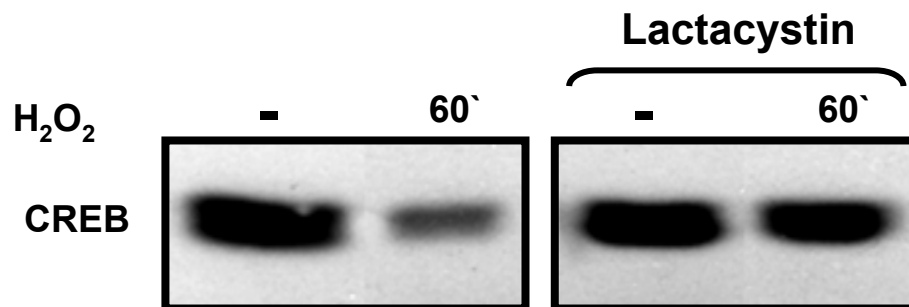


Figure 4

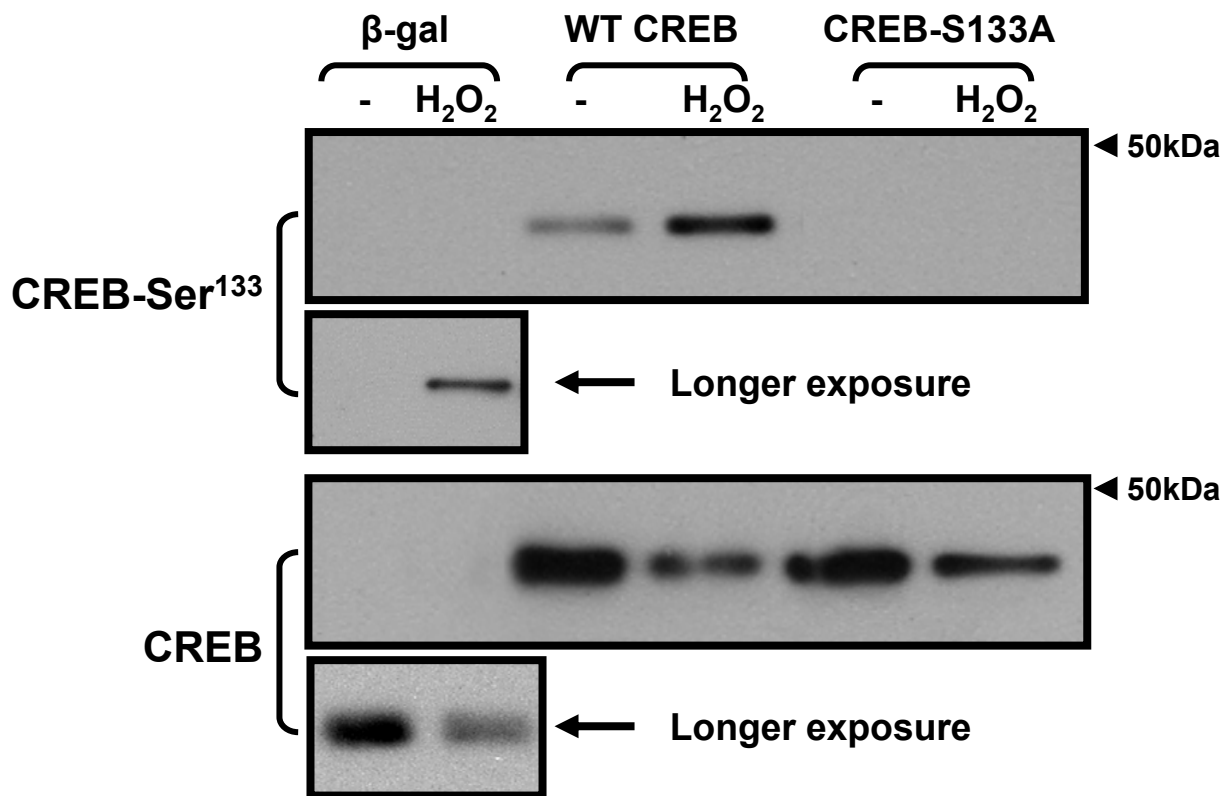


Figure 5

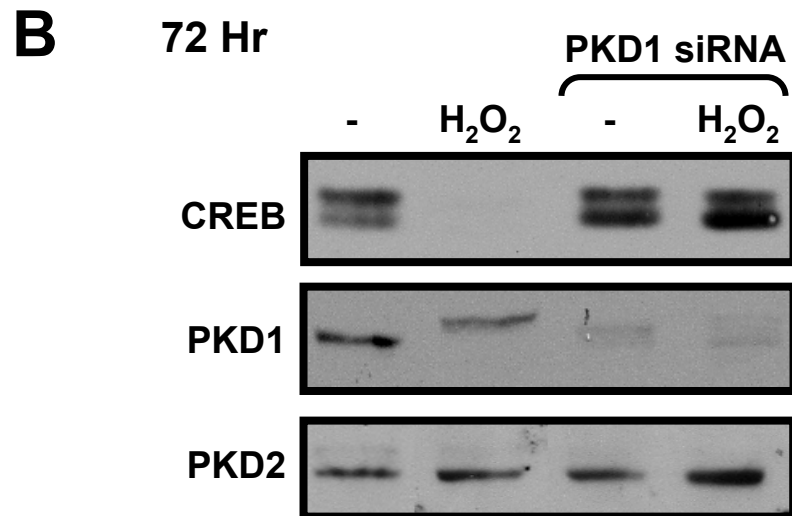
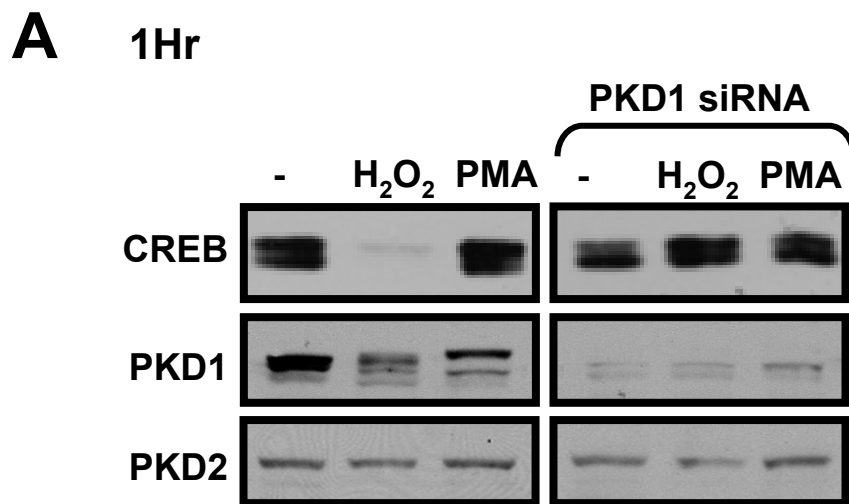


Figure 6