

Prevention of PKG1 α oxidation augments cardioprotection in the stressed heart

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The cGMP-dependent protein kinase-1 α (PKG1 α) transduces NO and natriuretic peptide signaling; therefore, PKG1 α activation can benefit the failing heart. Disease modifiers such as oxidative stress may depress the efficacy of PKG1 α pathway activation and underlie variable clinical results. PKG1 α can also be directly oxidized, forming a disulfide bond between homodimer subunits at cysteine 42 to enhance oxidant-stimulated vasorelaxation; however, the impact of PKG1 α oxidation on myocardial regulation is unknown. Here, we demonstrated that PKG1 α is oxidized in both patients with heart disease and in rodent disease models. Moreover, this oxidation contributed to adverse heart remodeling following sustained pressure overload or Gq agonist stimulation. Compared with control hearts and myocytes, those expressing a redox-dead protein (PKG1 α ^{C42S}) better adapted to cardiac stresses at functional, histological, and molecular levels. Redox-dependent changes in PKG1 α altered intracellular translocation, with the activated, oxidized form solely located in the cytosol, whereas reduced PKG1 α ^{C42S} translocated to and remained at the outer plasma membrane. This altered PKG1 α localization enhanced suppression of transient receptor potential channel 6 (TRPC6), thereby potentiating antihypertrophic signaling. Together, these results demonstrate that myocardial PKG1 α oxidation prevents a beneficial response to pathological stress, may explain variable responses to PKG1 α pathway stimulation in heart disease, and indicate that maintaining PKG1 α in its reduced form may optimize its intrinsic cardioprotective properties.

Introduction

Cyclic GMP (cGMP) and its principal effector PKG transduce NO and natriuretic peptide signaling to regulate cardiac and vascular function (1). In the heart, PKG activation suppresses adverse responses to a broad range of pathological cardiac stresses (2–4). These benefits have driven efforts to leverage PKG signaling for the treatment of heart failure, activating the kinase by stimulating cGMP synthesis, reducing natriuretic peptide proteolysis, and/or blocking cGMP hydrolysis (5, 6).

Despite enthusiasm for the therapeutic potential of PKG enhancement strategies, their impact has been somewhat variable, raising concerns that cGMP/PKG signaling may become blunted by disease modifiers such as oxidative stress (7), which depresses NO (7) and cGMP synthesis (8, 9). Studies have also shown that oxidative stress impacts PKG1 α , the primary cardiovascular isoform. PKG1 α is a homodimeric serine/threonine kinase containing protein interactive (leucine zipper), autoinhibitory, and cGMP-binding domains in the N terminus and a catalytic domain in the C terminus (10). Classical activation occurs upon cGMP binding to regulatory sites, releasing autoinhibition; however, the kinase can also be stimulated by oxidation at cysteine 42 (C42), forming an intermolecular disulfide bond (11). As this cysteine lies at the proximal end of the leucine zipper, it may also influence protein interactions and localization. In resistance arteries, PKG1 α oxidation transduces endothelium-derived hyperpolarizing factor to induce vasorelax-

ation (11, 12), and mice harboring a knockin redox-dead mutation (PKG1 α ^{C42S}, abbreviated herein as KI) develop modest hypertension (12). Here, we reveal that in the heart, PKG1 α oxidation impairs its cardioprotective effects against pathological stress. This is linked to redox-sensitive changes in intracellular localization and protein targeting of the kinase, diminishing the ability of PKG1 α to counter prohypertrophic and profibrotic pathways.

Results and Discussion

PKG1 α C42 oxidation occurs in heart disease and worsens the cardiac response to pressure overload. PKG1 α oxidation increased in human ischemic heart failure (Figure 1A and see patient characteristics in Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI80275DS1), and similar increases were observed in mouse hearts exposed to sustained pressure overload (Supplemental Figure 1A) and in canine dilated cardiomyopathy (Supplemental Figure 1B). Hearts expressing PKG1 α ^{C42S} only formed the monomer (Supplemental Figure 1A), confirming that its appearance in nonreducing gels indeed reflects C42 dimerization. To test whether PKG1 α oxidation alters the cardiac response to stress, KI and littermate control mice were subjected to chronic transaortic constriction (TAC). TAC induced chamber dilation, contractile depression, ventricular hypertrophy, interstitial fibrosis, and lung edema, and all were blunted in PKG1 α ^{C42S}-KI mice (Figure 1, B and C, and Supplemental Figures 2 and 3). The blunted organ-level structural and functional disease was accompanied by suppressed fetal gene reexpression (Supplemental Figure 4) and blunted pathological signaling cascades, including calcineurin/nuclear factor of activated T cells (Cn/NFAT), calcium/calmodulin-activated kinase II

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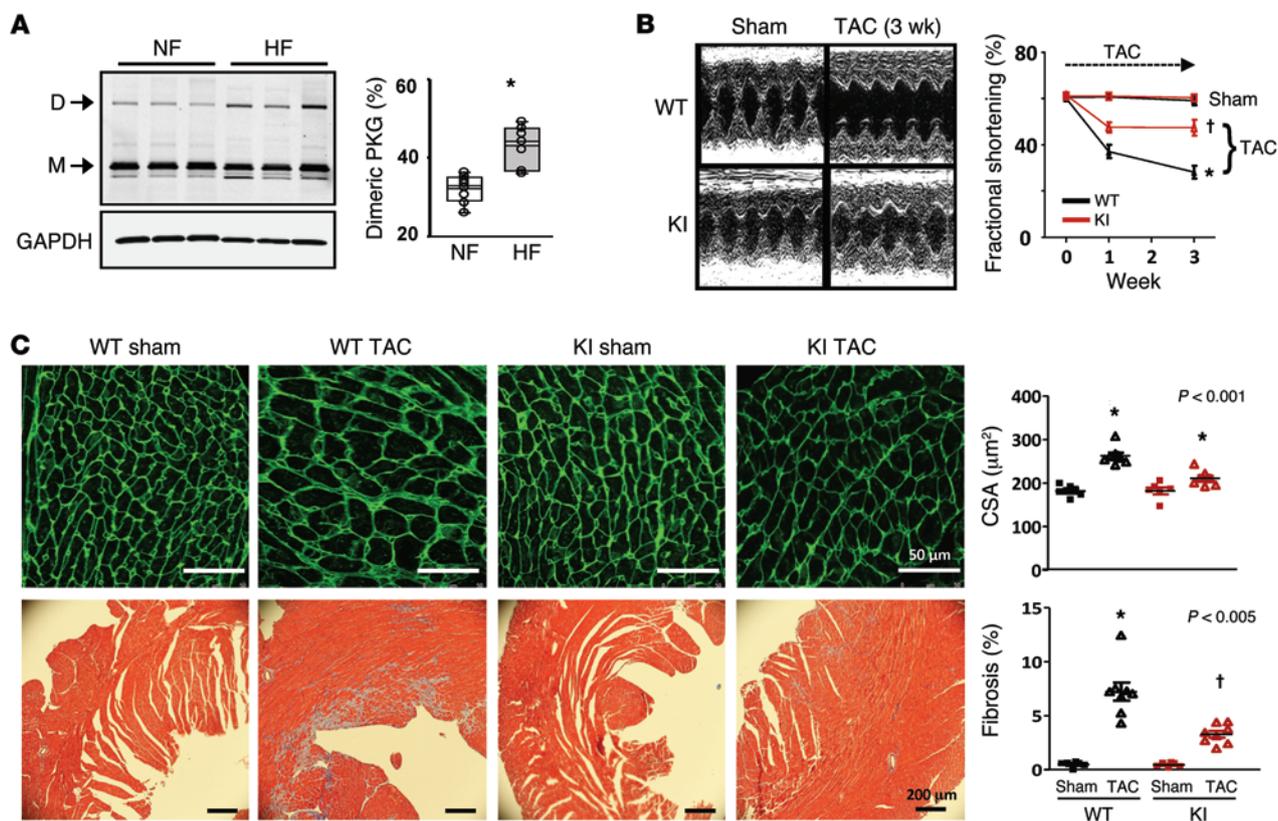


Figure 1. PKG1 α oxidation at C42 increases in failing and hypertrophied myocardium, and its prevention protects the heart against pressure overload.

(A) PKG1 α disulfide dimer formation was increased in human heart failure ($n = 7-8/\text{group}$, $*P < 0.001$). D, PKG1 α dimer; HF, heart failure; M, PKG1 α monomer; NF, nonfailing. (B) An example of M-mode echocardiograms from PKG1 α^{C42S} and littermate controls subjected to TAC and summary data for fractional shortening ($n = 9-10$, WT sham; $n = 17-19$, WT TAC; $n = 9-10$, KI sham; $n = 20-23$, KI TAC). Sham data were superimposable and thus combined for 2-way ANOVA interaction between TAC and genotype; ANOVA results: $*P < 0.005$ versus sham and KI TAC; $^{\dagger}P < 0.01$ versus sham. (C) Histopathology (wheat germ agglutinin [WGA] and Masson's trichrome) shows larger heart and myocyte size and fibrosis in WT versus PKG1 α^{C42S} after TAC. Left ventricular cross-sectional myocyte area and interstitial fibrosis ($n = 6$, sham; $n = 8$, TAC). $*P < 0.001$ versus baseline; $^{\dagger}P < 0.01$ versus baseline. P values show 2-way ANOVA interaction between TAC and genotype. CSA, cross-sectional area.

(CaMKII), AKT, and ERK1/2 (Figure 2, A-D). Cardioprotection occurred without an overall decline in oxidative stress, as the myocardial ratio of reduced glutathione/oxidized glutathione and lipid peroxidation (malondialdehyde [MDA] and 4-hydroxyalkenals [4-HAE]) were similar in both genotypes (Figure 2E).

Myocyte PKG1 α oxidation controls its sarcolemmal membrane translocation. While previous studies predicted that PKG1 α oxidation would increase activity (12), an in vitro kinase assay analysis yielded similar increases in activity in both WT and KI TAC myocardium (Supplemental Figure 5A). The lack of apparent enhanced activity despite PKG1 α oxidation may be due in part to assay limitations (see Supplemental Methods) but most likely relates to higher cGMP levels (Supplemental Figure 5B) that blunt oxidative activity change (11, 13). This effect was confirmed in isolated myocytes exposed to 10 minutes of H₂O₂ (10 μM , dose response in Supplemental Figure 6A), where increased in vitro activity was detected if cGMP levels were low, but not when they became elevated (Supplemental Figure 6B). Expression of PKG1 α^{C42S} prevented H₂O₂-stimulated activation (Supplemental Figure 6, B and C).

An alternative mechanism for altering PKG1 α functionality is a change in intracellular localization. IHC showed that both WT and C42S PKG1 α proteins were diffusely distributed in adult myocytes at

baseline. However, after a 10-minute exposure to H₂O₂, both forms of PKG1 α translocated to the sarcolemmal membrane (Figure 3A), whereas after 1 to 2 hours of exposure, WT PKG1 α was again cytosolic, while PKG1 α^{C42S} remained predominantly at the membrane. This disparity was also observed in myocytes isolated from 3-week-old TAC hearts (Figure 3B). PKG1 α relocalization was confirmed by subfractionation immunoblots (Supplemental Figure 7), in which the H₂O₂-induced (60 minutes) PKG1 α dimer appeared only in the cytosol, whereas the reduced monomer appeared in both the cytosol and membrane. This disparity was unaltered by blocking PKG1 α activity with DT3, so it was independent of kinase activity.

Coupling of differential localization of PKG1 α to antihypertrophic molecular targeting. Plasma membrane-localized PKG1 α^{C42S} could potentially augment antihypertrophic signaling by enhancing its targeting of disease-related proteins at this location. We tested this in neonatal rat cardiomyocytes (NRCMs) expressing WT or C42S-mutant PKG1 α (adenoviral infection) that were then exposed to 48 hours of endothelin 1 (ET1) or phenylephrine (PE). The dominant form of PKG1 α was provided by the transgene, and this cellular model recapitulated the in vivo findings, including lack of dimer formation (Supplemental Figure 8), sustained plasma membrane localization, and suppression of myocyte hypertrophic

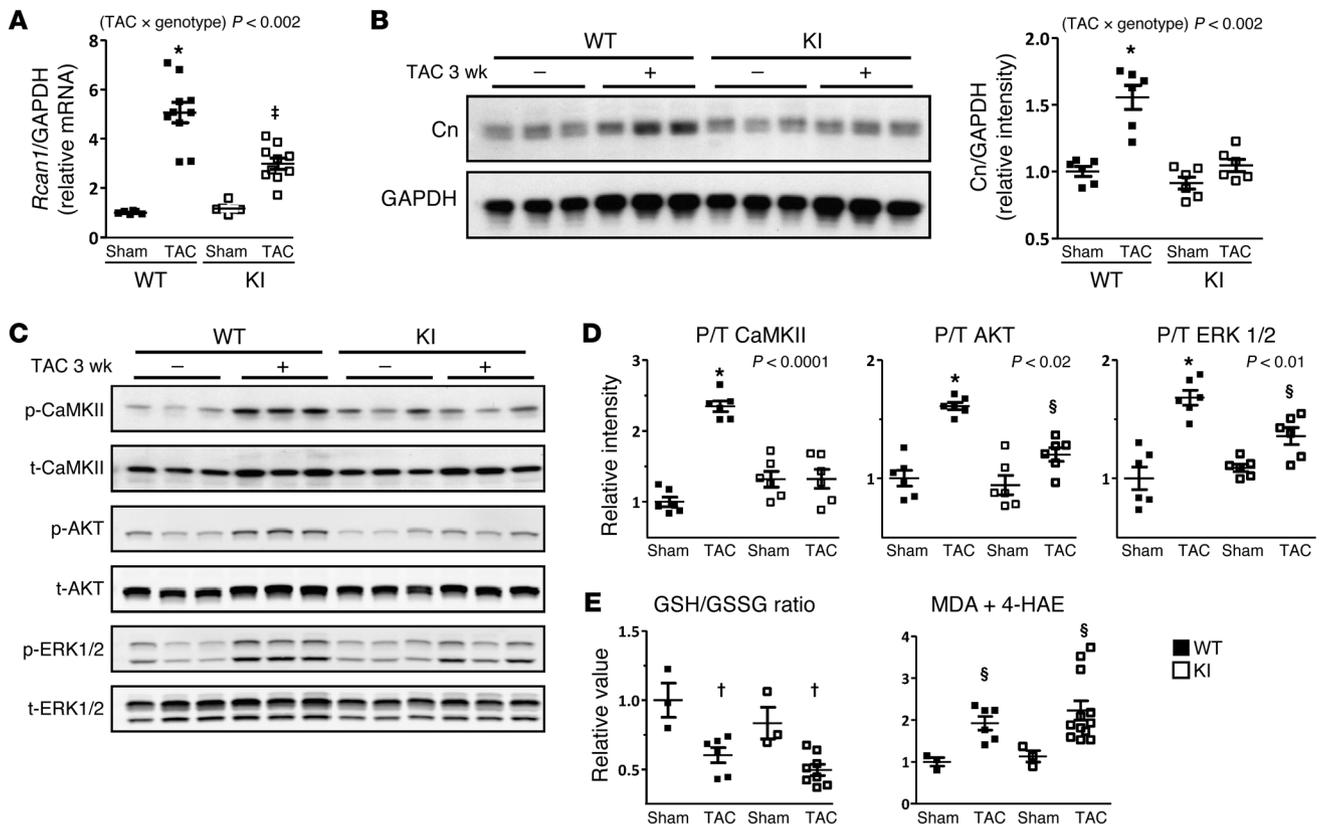


Figure 2. Prevention of PKG1 α C42 oxidation blocks maladaptive signaling due to pressure overload, without altering global myocardial oxidative stress. (A) Gene expression of regulator of calcineurin 1 (*Rcan1*) and (B) protein levels of Cn. (C) Representative gels for phosphorylated and total proteins for CaMKII, AKT, and ERK1/2 in sham and 3-week-old TAC hearts and (D) summary data ($n = 6$ /group). (E) Ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) ($n = 6$, WT; $n = 8$, KI) and MDA and 4-HAE ($n = 6$, WT; $n = 12$, KI). * $P < 0.0001$, † $P < 0.01$, ‡ $P < 0.001$, and § $P < 0.05$ versus respective controls. P values shown relate to the genotype interaction effect on the TAC-stimulated response. p-, phosphorylated; t-, total; TAC \times genotype, interaction between TAC and genotype.

signaling in PKG1 α^{C42S} -expressing cells (Supplemental Figures 9 and 10). Enhanced cytoprotection was lost by adding DT3, confirming the need for PKG activation. As in the myocardium, cellular PKG1 α activity measured in vitro was similar in cells expressing WT or PKG1 α^{C42S} protein (Supplemental Figure 11).

We next tested whether membrane-localized PKG1 α -targeted proteins were preferentially impacted by PKG1 α^{C42S} , focusing on 3 known candidates. Regulator of G protein signaling (RGS) RGS2 and RGS4 bind to and are activated by PKG1 α , blunting the hypertrophy and dilation otherwise induced by *Gaq* activation and mechanical stress (14, 15). To test whether reduced PKG1 α favored RGS2/4 activation, one or both RGS genes were suppressed by siRNA (Supplemental Figure 12A). However, the relative protective response from C42S PKG1 α persisted, regardless of RGS2 or RGS4 suppression (Supplemental Figure 12B), and the PKG1 α redox state did not impact protein PKG1 α -RGS2 binding (Supplemental Figure 12C); thus, this was not the mechanism. A third major candidate is the transient receptor potential canonical channel type 6 (TRPC6), which is activated by *Gaq*-coupled stimuli to trigger Cn/NFAT signaling (16). TRPC6 is also phosphorylated by PKG, blunting its conductance and stimulation of hypertrophy and fibrosis (17, 18). To test its role, NRCMs were incubated with a selective TRPC3/6 antagonist (GSK503A). ET1-stimulated hypertrophic signaling was substantially blocked by GSK503A

(Figure 3C and Supplemental Figure 13A) in controls, whereas cells expressing PKG1 α^{C42S} had a blunted ET1 response that was not further altered by adding GSK503A ($P < 0.001$ for interaction between GSK503A treatment and the PKG1 α genotype). TRPC6 inhibition also reduced Cn, CaMKII, and ERK1/2 activation (Figure 3D and Supplemental Figure 13B) in WT PKG1 α -expressing cells, but had less effect in cells expressing PKG1 α^{C42S} ($P < 0.01$ for interaction). These results indicated that TRPC6-dependent signaling was blunted more if PKG1 α oxidation was prevented.

PKG1 α phosphorylates TRPC6 at T70 and S322 (for human; T69 and S321 for mouse) (18). Accurate detection of either modification in myocardium or myocytes remains difficult because of assay limitations. As an alternative, myocytes were coinfecting with either WT TRPC6 or a phosphosilenced mutant (TRPC6^{T70A,S322Q}), the latter preventing TRPC6 modulation by PKG (18). Cells expressing either form of PKG1 α were then exposed to ET1. Baseline hypertrophic gene expression was similar in all groups (Figure 3E), and ET1 induced similar increases in cells expressing WT PKG1 α , regardless of the form of TRPC6. However, TRPC6^{T70A,S322Q} selectively increased ET1 responses in PKG1 α^{C42S} -expressing cells, returning them toward WT PKG levels ($P < 0.01$, PKG1 α and TRPC6 genotype interaction). Thus, PKG1 α C42 oxidation blunts its targeting and suppression of TRPC6 and associated signaling, whereas keeping PKG1 α reduced more effectively blocks the pathway.

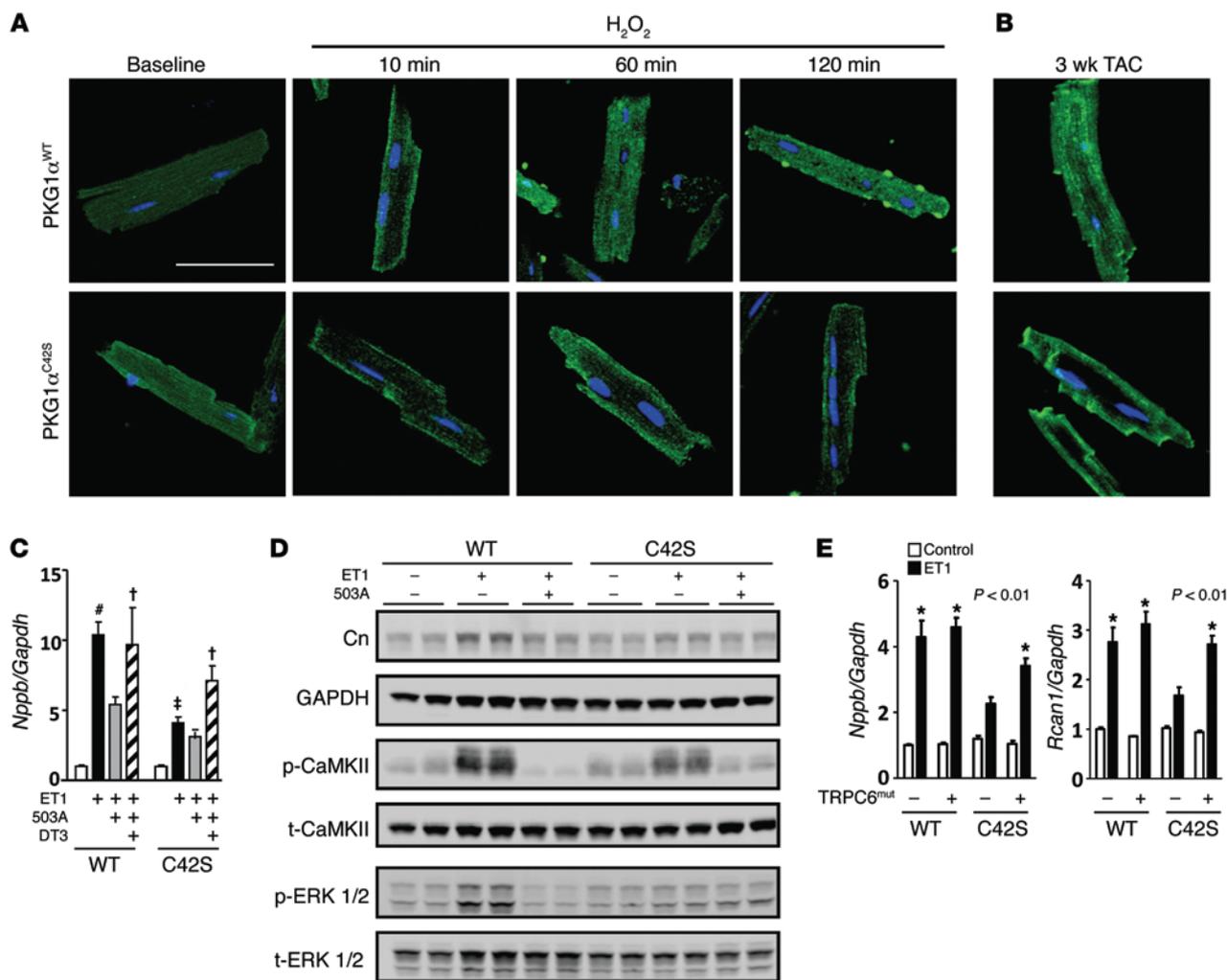


Figure 3. Protective effects of prevention of PKG1 α C42 oxidation are related to changes in intracellular localization and TRPC6 targeting by the kinase. (A) Time-dependent myocyte PKG1 α plasma membrane translocation and rediffusion with 10 μ M H₂O₂ exposure in cells isolated from WT or PKG1 α ^{C42S} hearts, representative of 3 experiments. Scale bar: 100 μ m. (B) Localization disparities in PKG1 α myocytes from TAC hearts in WT versus C42S-KI mice, representative of 30 cells (2 hearts) for each group. (C) Hypertrophic signaling from ET1 was blunted by TRPC3/6 blockade (503A) in NRCMs expressing WT PKG1 α , but not when PKG activity was blocked by DT3. 503A did not alter the response in PKG1 α ^{C42S} cells, whereas DT3 reversed the protection from C42S-mutant PKG1 α cells. #*P* < 0.001 versus control and ET1 + 503A; †*P* < 0.05 versus ET1 + 503A; ‡*P* < 0.05 versus control. *P* values for 2-way ANOVA interaction between ET1 and genotype, *P* < 0.001; interaction between ET1 + 503A and genotype, *P* < 0.01. *n* = 7, control; *n* = 7–8, ET1; *n* = 6, ET1 + 503A; *n* = 3, ET1 + 503A + DT3. (D) Western blot for Cn expression and CaMKII and ERK1/2 phosphorylation after 48 hours of ET1 stimulation, with or without TRPC3/6 inhibition, in NRCMs (*n* = 6/group). See Supplemental Figure 12B for summary data. (E) NRCMs expressing PKG1 α ^{WT} or PKG1 α ^{C42S} and TRPC6^{WT} or TRPC6^{T70A,S322Q} (TRPC6^{mut}). In PKG1 α ^{C42S}-expressing cells, ET1 gene induction was blunted, but this was reversed by coexpressing TRPC6^{mut}. *n* = 6/group. *P* values for 2-way ANOVA interaction between ET1 and TRPC6 (or PKG) genotype, **P* < 0.001 versus respective controls.

This study reveals what we believe to be a novel mechanism for oxidative stress-mediated dysfunction in the stressed heart and myocytes involving PKG1 α oxidation and consequent changes in its intracellular targeting. This differs from a net gain of function from the PKG1 α oxidation observed in resistance vessels (12, 13) that has been attributed to cGMP-independent kinase activity. The latter is based primarily on a purified protein kinetics assay (11) revealing increased substrate-binding affinity from oxidation. However, oxidative activation was reduced when cGMP was increased (11, 13), likely due to physical separation of N terminus domains following the binding of cGMP to its regulatory sites (19). Here, we reveal an alternative mechanism for PKG1 α functionality, namely a change in intracellular targeting that is likely due to

modification of leucine zipper domain interactions (20). This concept is supported by results in KI mice expressing a PKG1 α leucine zipper-deficient protein, which display worsened stress cardiomyopathy (21) despite unaltered in vitro PKG kinase activity (22).

Membrane translocation of PKG1 α occurs in smooth muscle (23) and cardiomyocytes (14), where it facilitates suppression of Gq-coupled signaling by RGS2 and RGS4. However, this relocation and protection is transient (14), and the current results show how PKG1 α oxidation could explain this time course. Though we did not find favored interactions between PKG1 α ^{C42S} and RGS2 or RGS4, results supporting enhanced targeting of TRPC6 were obtained. In addition to Gq-coupled (17, 18) and mechanical stimulation (24), TRPC6 is indirectly stimulated by

oxidative stress that involves oxidation of another previously unidentified intracellular protein (25). Reduced channel suppression by PKG1 α oxidation fits this observation. Redox sensitivity of PKG1 α suppression of TRPC6 may also have therapeutic implications beyond the heart, including in dystrophinopathies (24) and glomerulosclerosis (26, 27), where ROS and TRPC6 play a pathophysiological role. Future studies are needed to identify the protein partners that control PKG1 α translocation and develop strategies to selectively sustain its reduced state to maximize the efficacy of its activation against myocardial disease.

Methods

Mice expressing the PKG1 α C42S mutant as a global KI were generated on a C57Bl/6 background as previously described (12) and compared with littermate controls. Pressure overload was induced by TAC (3), with serial echocardiography used to assess intact heart function. Postmortem tissue was analyzed for molecular signaling and histology. A cell-based model was also used, involving isolated NRMCs infected with adenovirus expressing either WT or C42S-mutant PKG1 α .

Statistics. Statistical analysis included 1- or 2-way ANOVA, analysis of covariance, and paired tests with nonparametric analysis when required. Complete experimental and statistical methods are provided in the supplemental materials.

Study approval. All procedures were performed in accordance with institutional and NIH guidelines for the care and use of animals in biomedical research and were approved by the IACUC of the Johns Hopkins Medical Institutions.

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