PKG\(\alpha\) is activated by metal-dependent oxidation \textit{in vitro} but not in intact cells

Type I cGMP-dependent protein kinases (PKGIs) are important components of various signaling pathways and are canonically activated by nitric oxide– and natriuretic peptide– induced cGMP generation. However, some reports have shown that PKG\(\alpha\) can also be activated \textit{in vitro} by oxidizing agents. Using \textit{in vitro} kinase assays, here, we found that purified PKG\(\alpha\) stored in PBS with Flag peptide became oxidized and activated even in the absence of oxidizing agent; furthermore, once established, this activation could not be reversed by reduction with DTT. We demonstrate that activation was enhanced by addition of Cu\(^{2+}\) before storage, indicating it was reduced. In contrast, under the same conditions, purified PKG\(\beta\) activity only slightly increased with storage. Using PKG\(\alpha\)/PKG\(\beta\) chimeras, we found that residues throughout the PKG\(\alpha\)-specific autoinhibitory loop were responsible for this activation. To explore whether oxidants activate PKG\(\alpha\) in H9c2 and C2C12 cells, we monitored vasodilator-stimulated phosphoprotein phosphorylation downstream of PKG\(\alpha\). While we observed PKG\(\alpha\) Cys\(^{43}\) crosslinking in response to H\(_2\)O\(_2\) (indicating an oxidizing environment in the cells), we were unable to detect increased vasodilator-stimulated phosphoprotein phosphorylation under these conditions. Taken together, we conclude that while PKG\(\alpha\) can be readily activated by oxidation \textit{in vitro}, there is currently no direct evidence of oxidation-induced PKG\(\alpha\) activation \textit{in vivo}.

The type I cGMP-dependent protein kinases (PKG) play important roles in diverse physiological and pathophysiological processes. Their most studied and best understood signaling functions are in the cardiovascular system, where they control cardiac myocyte and smooth muscle contractility, but they also play key roles in synaptic plasticity, bone regulation, and beige/brown fat differentiation (1–3). As a result of differential splicing, mammalian cells express two PKG isoforms, PKG\(\alpha\) and PKG\(\beta\), which have unique N-terminal leucine zipper and autoinhibitory domains, but identical cyclic-nucleotide binding and catalytic domains (4, 5). The unique N-terminal domains cause PKG\(\alpha\) and PKG\(\beta\) to form homodimers, target the kinases to different substrates, and cause PKG\(\alpha\) to have a higher affinity for cGMP than PKG\(\beta\) (6, 7). The higher cGMP affinity in PKG\(\alpha\) correlates with a lower activation constant \((K_a)\) for cGMP (6).

While the PKGI enzymes are canonically activated downstream of nitric oxide– and natriuretic peptide– induced cGMP generation, various groups have reported oxidation-induced direct activation of the kinase (3–7). The first report was by Landgraf et al. (8), where the authors demonstrated that PKG\(\alpha\) was activated by oxidation in the presence of various metal ions. Using tryptic digests and mass spectrometry, they identified Cys\(^{118}\), Cys\(^{196}\), Cys\(^{313}\), and Cys\(^{519}\) as the cysteines most likely mediating this effect. In 2007, Bugoyne et al. (9) reported that PKG\(\alpha\) could be activated by hydrogen peroxide (\(\text{H}_2\text{O}_2\))-induced disulfide formation between two cysteines at position 43 located at the C-terminal end of the leucine zipper/dimerization domain. However, we subsequently used cell-based and \textit{in vitro} kinase assays to demonstrate that disulfide formation at Cys\(^{43}\) does not lead to PKG\(\alpha\) activation (10). We also found that the C43S mutation, which was generated to produce a “redox-dead” PKG\(\alpha\), caused PKG\(\alpha\) to have an approximately fivefold lower sensitivity to cGMP-induced activation \textit{in vitro}, compared to the WT enzyme (10). Our results were confirmed by Sheehe et al. (11). In addition, Sheehe et al. (11) concluded that \(\text{H}_2\text{O}_2\)-induced PKG\(\alpha\) activation was due to conversion of Cys\(^{43}\) to sulfonic acid and proposed that the negatively charged sulfonic acid interacted with basic residues distal to the autoinhibitory sequence.

During our previous studies, we found that cGMP-independent basal activity of purified Flag-epitope–tagged PKG\(\alpha\) increased after overnight storage in PBS with 100 ng/ml Flag peptide (Fig. 1). This activation occurred without the addition of an oxidizing agent and was associated with increased Cys\(^{43}\) crosslinking between the two peptide chains; however, while addition of DTT to the preactivated enzyme reversed Cys\(^{43}\) crosslinking, it did not reverse the increase in basal activity. The following studies were performed to probe the mechanism of PKG\(\alpha\) activation, under these conditions, and to assess whether this activation mechanism is physiologically important.
**Results**

**PKGIα basal activity increases after overnight storage in Flag elution buffer**

Freshly prepared Flag-tagged PKGIα was diluted in KPE [10 mM potassium phosphate and 1 mM EDTA (pH 7.0)] buffer alone or KPE with 5 or 15 mM DTT. Immediately before performing activity assays, samples of the diluted kinases were added to SDS sample buffer containing 100 mM maleimide, and the amount of Cys43-crosslinked PKGIα was determined by nonreducing SDS-PAGE (Fig. 1A). The kinase was approximately 42% crosslinked in the absence of DTT and the crosslinking was almost completely reversed by DTT. We measured kinase activity on the diluted samples and found that, compared to the maximum cGMP-stimulated activity, basal activity was 4.3 ± 0.69% in the absence of DTT and 3.4 ± 1.2% or 2.7 ± 0.69% when incubated with 5 or 15 mM DTT, respectively (Fig. 1B). The slightly lower basal activity in the presence of DTT is similar to our previous results (10). The purified PKGIα was then stored at 4°C overnight in elution buffer (PBS + 100 μg/ml Flag peptide). The next day, aliquots

---

**Figure 1. PKGIα basal activity increases after overnight storage in Flag elution buffer.** A, newly purified PKGIα was incubated for 1 h on ice in KPE buffer with the indicated amount of DTT and the level of Cys43-crosslinked PKGIα was determined by Western blotting (M = monomer, D = crosslinked dimer). B, kinase activity in the absence and presence of 10 μM cGMP was measured shortly after purification using an in vitro assay. C and D, the purified PKGIα was stored overnight at 4°C in elution buffer and then incubated for 1 h with the indicated amounts of DTT in KPE buffer. The amount of crosslinked PKGIα with Cys43 oxidized was determined by Western Blotting (C) and kinase activity was measured (D). E and F, in vitro kinase activity of newly purified PKGI (E) and after overnight storage with different levels of dilution in PBS (F). The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent protein preparations. PKGI, Type I cGMP-dependent protein kinase.

---
of the kinase were diluted in KPE buffer, with and without DTT, and kept on ice for 1 h. Western blots demonstrated that PKGα diluted in KPE in the absence of DTT was completely oxidized with ~75% migrating as a crosslinked dimer and ~25% running as an unknown oxidation product at a higher apparent molecular weight than the reduced monomeric protein (Fig. 1C). In the presence of 5 or 15 mM DTT, both oxidation products were reduced to ~40% or ~60% monomeric/reduced, respectively. The basal kinase activity was increased to a similar extent under all three conditions (Fig. 1D). These results are consistent with our previous finding that PKGα activity is independent of Cys43 cross-linking but demonstrates that the kinase is activated by some modification that is not easily reversed with DTT. Importantly, this modification occurred without adding H2O2 or other oxidizing agents to the purified protein (a second experiment with similar results is shown in Fig. S1).

It should be noted that the increase in basal kinase activity after overnight storage varied between different kinase preparations. This difference may be in part due to variable amounts of PKGα in each preparation and thus the ratio of protein to buffer during storage. To test this hypothesis, we purified PKGα and stored it overnight undiluted or diluted in elution buffer. As seen in Figure 1, E and F, the basal activity of a fresh PKGα preparation was 4.7 ± 0.80% and increased to 7.8 ± 0.32% after overnight storage when not diluted. However, when aliquots of this preparation were diluted to 2- and 5-fold before storage, the basal activity increased to 16 ± 1.4% and 39 ± 0.57%, respectively. Importantly, adding 2-fold more Flag peptide to the elution buffer had no effect on the increased activity, indicating that activation was not being mediated by the peptide (data not shown). Therefore, given the variability in the level of PKGα activation between protein preparations, all experiments in the main body of this article are from kinase reactions performed in triplicate on single protein preparations. To demonstrate qualitative reproducibility of the results, duplicate experiments using separate protein preparations are shown in Supplemental Data.

PKGα activation is prevented in the presence of reducing agents and metal chelators

Since short-term incubation with DTT did not reverse the kinase activation that had occurred during overnight storage, and metals have been shown to activate PKGα, we assessed whether activation could be prevented by adding either DTT or the metal chelator EDTA before overnight storage. As seen in Figure 2A, the basal activity of newly purified PKGα was 6.2 ± 0.34% and increased to 53 ± 0.97% after overnight storage in elution buffer alone, but in samples stored in elution buffer with DTT or EDTA, the increase in basal activity was largely prevented (6.0 ± 0.53 and 9.1 ± 0.58%, respectively) (Fig. 2B). To directly test the effect of heavy metals, we measured the basal activity of newly purified PKGα and then stored it overnight with and without added Cu2+. Basal activity of newly prepared kinase was 11 ± 1.6% of maximum and increased to 36 ± 0.62% versus 61 ± 2.2% after overnight storage in the absence or presence of added Cu2+, respectively (Fig. 2C). Taken together, these results are consistent with oxidation-induced activation being driven by the presence of trace metals in the storage buffer.

PKGα activation is independent of Cys43 oxidation

Even though Cys43 crosslinking was not directly responsible for PKGα activation, it is still possible that Cys43 plays role in the observed activation. Therefore, we compared activation between WT and C43S PKGα. The basal activity of newly purified WT and C43S PKGα were 4.6 ± 1.8 and 6.1 ± 3.2%, respectively (Fig. 3A). The amount of crosslinked WT PKGα was ~49% and as expected, no crosslinking was seen in the C43S mutant (Fig. 3B). After overnight storage, basal activity of WT and C43S PKGα increased to a similar extent, 33 ± 0.91 and 31 ± 0.50% of maximum activity, respectively (Fig. 3C). Similar results are shown in Fig. S3. The WT enzyme was completely crosslinked at Cys43 (Fig. 3D); however, it should be noted that the crosslinked WT and the monomeric C43S PKGα bands appeared as doublets, suggesting that oxidation events beyond Cys43 crosslinking were occurring. Similar doublets have been reported by Donzelli et al. (12) and are thought to be the result of disulfide bond formation between Cys118 and C196.

Prysyazhna et al. (13) reported that Cys43 crosslinking alters PKGα’s activation by cGMP; however, in a previous study, we found that Cys43 crosslinking had no effect on the $K_a$ for

![Figure 2](image-url) **PKGα activation is prevented in the presence of reducing agents and metal chelators.** A, in vitro kinase activity of PKGα within 1 h of purification. B, PKGα activity after overnight incubation in elution buffer alone or in elution buffer with the addition of DTT or EDTA, as indicated. C, PKGα activity when freshly prepared and after overnight storage ±200 μM CuCl2. The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed in two separate experiments. PKG1, Type I cGMP-dependent protein kinase.
Oxidation activates PKGα in vitro but not in cells

![Figure 3. PKGα activation is independent of C43S oxidation.](image)

The activities of freshly purified WT and C43S PKGα were analyzed by in vitro kinase assays. Western blotting showing amount of Cys43-crosslinked PKGα in the two preparations immediately after purification (M = monomer, D = crosslinked dimer). C, In vitro kinase assays performed using the protein preparations from (A) after overnight storage in elution buffer. D, Western blot showing the amount of crosslinked PKGα after overnight storage. The figure data shows from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent protein preparations. PKGα, Type I cGMP-dependent protein kinase.

PKGIβ is not highly activated by overnight oxidation

Since PKGIα and PKGIβ have identical sequences in their first cyclic nucleotide-binding pockets (which contain both Cys118/Cys133 and Cys196/Cys311), we examined whether PKGIβ is also activated during overnight storage. PKGIα and PKGIβ purified and immediately assayed showed a basal activity of 4.9 ± 1.2% and 1.6 ± 0.54% of maximum, respectively (Fig. 5A). After overnight storage at 4 °C, as expected, the basal activity of PKGIα increased to 21 ± 1.3%, whereas the basal activity of PKGIβ only slightly increased to 4.0 ± 0.78%. Similar results are shown in Fig. S6, A and B. These findings are consistent with those reported by Sheehe et al. (11), who showed that unlike PKGIα, purified PKGIβ was resistant to H2O2-induced activation.

Testing the activation mechanism proposed by Sheehe et al.

To explain the different response of PKGIα and PKGIβ to H2O2-induced activation, Sheehe et al. (11) proposed a mechanism in which basic residues unique to the PKGIα autoinhibitory loop interacted with a negatively charged sulfonic acid moiety formed at Cys118 in response to H2O2. We tested this mechanism by mutating the basic residues found in the PKGIα autoinhibitory loop to the corresponding nonbasic residues in PKGIβ. Specifically, we simultaneously mutated PKGIA Arg52 to Phe (R82F) and Lys83 to Pro (K83P). We found that in freshly purified preparations, the basal activity of the mutant protein (referred to as RK/FP) was similar to WT PKGIα, and that the mutations did not prevent activation after overnight storage (Fig. 5, C and D). Similar results are shown in Fig. S6, C and D. These results are not consistent with the activation mechanism proposed by Sheehe et al., but suggest different mechanisms, tested below.

Residues throughout the PKGIα autoinhibitory region mediate oxidant-induced activation of PKGIα

Since overnight storage differentially affected the basal activities of PKGIα and PKGIβ, we made chimeric enzymes in which we swapped the leucine zipper domains between the two kinases (chimera C1, Fig. 6A). The α/β kinase had a PKGIα leucine zipper with a PKGIβ autoinhibitory domain and the β/α kinase had the opposite (the remaining sequences are identical between the two isoforms). We then performed

serine at the position analogous to Cys196 (Fig. 4C). Thus, we compared activation of WT, C118A, and C196V PKGIα. As seen in Figure 4D, the basal activities of WT, C118A, and C196V were 5.9 ± 1.4, 5.0 ± 0.9, and 6.9 ± 1.1% of maximum, respectively. The next day, basal activities increased to 53 ± 1.9 of maximum for WT but only to 17 ± 1.8 and 35 ± 0.1% for the C118A and C196V mutant kinases, respectively (Fig. 4E). Similar results for a separate enzyme purification are shown in Fig. S5. While mutation of Cys118 had the most pronounced effect on preventing activation, the C196V mutation also reduced the level of activation. Together, these data demonstrate that in addition to oxidation of Cys118, oxidation of Cys196 and/or other residues can also induce PKGIα activation.

Mutation of either Cys118 or Cys196 reduces oxidation-mediated PKGIα activation

In order to determine if oxidation of PKGIα Cys118 or Cys196 was responsible for the overnight activation, we used mutagenesis to change the cysteines to nonoxidizable residues. These residues are located in the first cGMP-binding pocket (Fig. 4A), and a disulfide bond was seen between these residues in a crystal structure of the isolated CNB-A/B domains [Fig. 4B and (16)]. Since we wanted to produce mutations that prevent oxidation-induced activation, but otherwise have no effect on basal kinase activity or cGMP response, we identified amino acid differences at these positions in homologous proteins, reasoning that changing the cysteines to these residues would be less likely to disrupt folding of the cGMP-binding pocket. Thus, we aligned PKGI, PKGII, and PKA R1α amino acid sequences and found that R1α has an alanine at the position analogous to Cys118 and that PKGII has a valine and R1α has a
in vitro kinase assays on freshly purified PKGIα, PKGIβ, PKGIα/β, and PKGIβ/α and found that they had similar basal activities (Figs. 6B and S7A). After overnight storage, the basal activities of PKGIα and PKGIβ/α increased to a similar degree, but the basal activities of PKGIβ and PKGIα/β remained low (Figs. 6C and S7B). Thus, activation required residues in the PKGIα autoinhibitory domain. To localize the residues responsible for activation, we made another set of complementary chimeric enzymes by swapping the amino acids N-terminal to the ISAEP amino acid sequence, which is conserved in both isoforms and located after the pseudosubstrate sequence in the autoinhibitory domain (chimera C2, Fig. 1A). After overnight storage, basal activity increased in both chimeric enzymes, but the increase was less than that seen for WT PKGIα (Fig. 6, D and E). The same pattern of activation was seen with separate enzyme preparations (Fig. S7, C and D), suggesting that activation is most likely mediated through an additive effect involving residues throughout the PKGIα autoinhibitory loop.

**Testing the effect of acidic residue mutations at PKGIα Cys^{118} and PKGIβ Cys^{196} on kinase activity**

Since Sheehe et al. demonstrated that H₂O₂ treatment caused conversion of Cys^{118} to a negatively charged acid moiety which then induces kinase activation, we examined the effect of mutating Cys^{118} to Asp. We also assessed the corresponding mutation in PKGIβ (i.e., C133D). Freshly purified C118D PKGIα and C133D PKGIβ had higher basal activities than the WT enzymes (Fig. 7A). The basal activities of both mutants further increased after overnight storage (Fig. 7B), indicating that the enzymes were activated by modification of one or more additional site(s). Separate enzyme preparations with similar results are shown in Fig. S8.
Oxidation activates PKGα in vitro but not in cells

H₂O₂ does not activate PKGα in cultured cells

The H9c2 cell line was originally derived from embryonic rat heart (17). The cell line expresses endogenous PKGα and vasodilator-stimulated phosphoprotein (VASP), a well-characterized PKG substrate. To assess how H9c2 cells respond to cGMP-induced PKGα activity, we treated the cells with increasing amounts of 8-pCPT-cGMP and monitored VASP phosphorylation reached at 30 μM 8-pCPT-cGMP within 1 h of purification. Fase assays performed with WT and R82F/K83P (RK/FP) PKGα within 1 h of purification. Similar results were observed with two independent enzyme purifications. PKGI, Type I cGMP-dependent protein kinase.

Metal-induced activation of purified PKGα in vitro

The first description of PKGI regulation by oxidation was reported by Landgraf et al., who found that PKGI purified from bovine lung could be activated by incubation with various metals, including Ag⁺, Hg⁺, Cu⁺, Cu²⁺, and Fe³⁺. They also demonstrated that activation by Cu²⁺ was blocked by co-incubation with the reducing agent DDT or the metal chelator EDTA (8). These results are consistent with our current findings, which suggest that trace metals in the buffers (or carried over from cell extracts during purification) induced PKGα activation during storage. These authors found that Cu²⁺-induced activation could be reversed by removing the Cu²⁺ by gel filtration and reducing the enzyme with DTT, and they concluded that this activation was due to the formation of intrachain disulfide bond(s) between either Cys¹¹⁸:Cys⁴³ or Cys¹¹³:Cys⁵¹⁹. Consistent with this conclusion, Donzelli et al. (12) proposed that PKGα could be activated by nitroxylic-induced disulfide bond formation between Cys¹¹⁸ and Cys⁴³, and Osborne et al. (16) observed a disulfide bond between these residues in a crystal structure of the PKGα cyclic nucleotide-binding domains. In contrast to activation being induced by formation of a Cys¹¹⁸:Cys⁴³ disulfide bond, Sheehan et al. found that H₂O₂-induced oxidation converted Cys¹¹⁸ to sulfonic acid and proposed that PKGα activation was caused by interaction between the newly formed acidic moiety and basic residues unique to the PKGα autoinhibitory domain. While our current results are consistent with the conversion of Cys¹¹⁸ to sulfonic acid, we found that mutation of the basic residues that were predicted to interact with the sulfonic acid moiety did not prevent PKGα activation.

Oxidant-induced PKGα crosslinking at Cys⁴³ does not increase kinase activity but may alter cellular targeting

In 2007, Burgoyne et al. (9) reported that PKGα could be activated by oxidant-induced disulfide formation between...
two cysteines at position 43 located at the end of the leucine zipper in each PKGIα peptide in the homodimer. A knock-in mouse containing PKGIα with a C43S mutation has a phenotype consistent with loss of PKGIα function, which implied oxidation-induced PKGIα activation was an important physiological mechanism for regulation the kinase (21–24). However, we and others have reported that Cys43 crosslinking does not increase PKGIα kinase activity in vitro (10, 11). Importantly, we found that the ‘redox-dead’ C43S mutation caused PKGIα to be 5-fold less sensitive to cGMP-induced activation (10). A decrease in cGMP sensitivity for C43S PKGIα was also seen by Shehee et al. (11). The reduced cGMP affinity could theoretically explain the loss-of-function phenotype of the C43S PKGIα knock-in mouse.

The PKGIα leucine zipper domain is involved in mediating homodimerization of the enzyme and also targets the kinase to specific substrates (25–27). The importance for proper PKGIα targeting in vivo has been demonstrated by a knock-in mouse with mutations in the leucine zipper that prevent dimerization. These mice show adult onset hypertension and are more sensitive to cardiac pressure overload than wild-type littermates (i.e., increased hypertrophy, systolic/diastolic dysfunction, and mortality) (28, 29). While PKGIα is dimeric in the absence of Cys43 crosslinking, crosslinking may stabilize the helical conformation of the leucine zipper, especially at its C-terminus, and may confine the conformation of an interface for protein–protein interactions (30). Consistent with this, Cys43 crosslinking increases the interaction between PKGIα
Oxidation activates PKG\(\alpha\) in vitro but not in cells

**Figure 7. Testing the effect of acidic residue mutations at PKG\(\alpha\) Cys\(^{118}\) and PKG\(\beta\) Cys\(^{156}\) on kinase activity.** A, kinase assays using newly purified PKG\(\alpha\), PKG\(\beta\), C118D PKG\(\alpha\), and C133D PKG\(\beta\) Assays were performed within 1 h of purification. B, kinase assays performed on the protein preparations shown in A after 20-h storage in elution buffer at 4 °C. The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent enzyme purifications. PKG\(\alpha\), Type I cGMP-dependent protein kinase.

and two of its known interacting proteins, MYPT1 and RhoA, in vitro (9).

**H\(_2\)O\(_2\) does not activate PKG\(\alpha\) in cardiac myocyte-derived H9c2 cells or C2C12 myoblasts**

The H9c2 cell line derived from embryonic rat hearts has been used as an alternative to primary cardiac myocytes (17). The cell line expresses PKG\(\alpha\) and VASP and thus serves as an ideal platform to study PKG\(\alpha\) signaling in a cellular context. VASP phosphorylation is a sensitive readout for PKG\(\alpha\) activation, and treating these cells with cell-permeable cGMP analogs leads to robust VASP phosphorylation. However, we were unable to detect VASP phosphorylation after treatment with relatively high amounts of \(\text{H}_2\text{O}_2\) in either H9c2 or C2C12 cells. While \(\text{H}_2\text{O}_2\) is an endogenous signaling molecule, the amounts found in vivo are thought to normally be in the low \(\mu\text{M}\) range but may reach higher levels under pathophysiological conditions (31). The finding that oxidant-induced PKG\(\alpha\) activation in vivo is due to irreversible modification of cysteines to sulfenic and/or sulfonic acids strongly argues against it serving as a dynamic signaling mechanism in vivo.

**H\(_2\)O\(_2\) may increase PKG\(\alpha\) substrate phosphorylation by activating soluble guanylate cyclase or inhibiting phosphatases**

If PKG\(\alpha\) is not activated by oxidation in intact cells, how are we to account for experiments showing that tissues from C43S knock-in mice are resistant to \(\text{H}_2\text{O}_2\) induced relaxation, but still relax in response to cGMP-analogs and nitrovasodilators? Previous studies have shown that treatment with \(\text{H}_2\text{O}_2\) can activate soluble guanylate cyclase (sGC) (32–34). This activation seems to require a reaction between \(\text{H}_2\text{O}_2\) and superoxide to form hydroxyl radicals (32) or metabolism of \(\text{H}_2\text{O}_2\) by catalase to form Compound I (33). However, sGC can also be inhibited by oxidation (35). Thus, treatment with \(\text{H}_2\text{O}_2\) may transiently activate sGC and produce a localized pool of cGMP. In this case, relaxation would rely on properly localized PKG\(\alpha\) with a high sensitivity to cGMP which can respond to this pool. Under such conditions, the loss of cGMP affinity and/or mislocalization of C43S PKG\(\alpha\) could explain the failure of tissues from the knock-in mouse to relax in response to \(\text{H}_2\text{O}_2\). It should be noted that H9c2 and C2C12 cells do not express sGC, since PKG\(\alpha\) is not activated in response to nitric oxide donors (data not shown).

An apparent increase in PKG\(\alpha\) activity may also be due to inhibition of serine/threonine phosphatases by \(\text{H}_2\text{O}_2\). Humphries et al. (36) found that enhanced cAMP-dependent protein kinase (PKA) substrate phosphorylation, seen when HeLa cells are treated with the sulfhydryl-specific oxidant diamide, is blunted in the presence of phosphatase inhibitors, indicating that the enhanced phosphorylation is due to phosphatase inhibition rather than kinase activation. While the exact phosphatases affected were not identified, PP1 and PP2A are known to dephosphorylate the PKA substrate CREB (37, 38), which is also a substrate for PKG\(\alpha\) (39). Interestingly, Kim et al. (40) found that \(\text{H}_2\text{O}_2\) treatment inhibits PP1 and PP2A in primary human diploid fibroblasts. Whether oxidant-induced phosphatase inhibition enhances PKG\(\alpha\) signaling in cells is currently unknown.

**Study limitations and future directions**

A limitation of this study is that in assessing the ability of oxidants to activate PKG\(\alpha\) in cells, we only examined one substrate (VASP) in two cell lines (H9c2 and C2C12). To analyze phosphorylation of other direct PKG\(\alpha\) substrates, we have tested a number of phospho-specific antibodies, but we found that they are not sensitive enough to detect substrate phosphorylation at endogenous protein levels in these cells. We have examined a number of primary cells and established cell lines, but we were unable to identify cells in addition to H9c2 and C2C12 cells which contain sufficient amounts of PKG\(\alpha\) without expressing sGC. Another
limitation of this study is that cell culture conditions may not reflect conditions found in vivo. It is possible that under certain pathophysiological conditions, which result in very high oxidant levels, PKGIα may become activated by oxidation-induced modification of Cys118 to an acid; but to our knowledge, there is no evidence that this modification occurs in cultured cells or in vivo. We are currently examining if Cys43 crosslinking changes PKGIα targeting in cells and the mechanism through which H2O2 may activate sGC.

Conclusion

In conclusion, the physiological significance of oxidation-induced PKGIα activation is doubtful. This is based on three main findings: (i) the observed in vitro oxidation is driven by metals in the presence of atmospheric oxygen; (ii) the activating modification is not easily reversed, arguing against a dynamic regulatory mechanism; and (iii) even in the presence of higher than physiological H2O2 levels, oxidant-induced PKGIα activation is not observed in cultured cells.

Experimental procedures

Materials

Fetal bovine serum, horseradish peroxidase (HRP)-conjugated anti-Flag M2 antibody, anti-Flag M2 affinity gel, and Flag peptide were from Sigma. Phospho-VASP (Ser239) Antibody was from Cell Signaling Technology. HRP-conjugated goat anti-mouse (115-035-062) and goat anti-rabbit (111-035-046)
antibodies were from Jackson Immuno Research. Kemptide was from AnaSpec, Inc. Cyclic nucleotide analogs were from BioLog Life Science Institute, and general laboratory reagents were from Fisher Scientific, Sigma Life Science, or Bio-Rad Laboratories.

**Vector constructs**

Flag-tagged WT PKGIA, WT PKGIB, and C43S PKGIA have been described previously (10). Additional mutations and chimeric PKGIA/PKGIB were produced using overlapping extension PCR (41, 42). PCR products were digested with BamHI and XhoI and ligated into BamHI/XhoI cut pFlag-D (10). All constructs derived by a PCR step were sequenced.

**Cell culture and transfection**

HEK293T/17 (ATCC ACS-4500), C2C12 (ATCC CRL-1772), and H9c2(2-1) (ATCC CRL-1446) cells were grown at 37 °C in a 5% CO2 atmosphere in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine 2000 Medium supplemented with 10% fetal bovine serum. Cells were transiently transfected into HEK293T cells and 24 h later, wells were treated with 8-CPT-cGMP or 5% milk. Cells were transfected using Lipofectamine2000 (Life Technologies) according to the manufacturer’s instructions.

**Kinase purification**

Flag-tagged WT and mutant PKGIA and PKGIB were purified as described (10). Briefly, Flag-tagged expression vectors were transiently transfected into HEK293T cells and 24 h later, cells were lysed in buffer A [PBS, 0.1% NP40, and protease inhibitor cocktail (Calbiochem #539131)]. Lysates were cleared by centrifugation and incubated with anti-Flag beads for 1 h at 4 °C. Beads were extensively washed, and PKG was eluted in PBS with 100 μg/ml Flag peptide. Purified kinases were either used immediately or assayed after overnight storage at 4 °C in elution buffer (~20 h). For some samples, kinases were diluted with an equal volume of PBS containing a two-fold concentration of added reagents (i.e., 30 mM DTT, 5 mM EDTA, or 200 mM Cu2+) before overnight storage.

**In vitro kinase assays**

Purified kinase was diluted to ~1 ng/μl in KPEB Buffer [10 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 0.1% bovine serum albumin]. For some reactions, KPEB contained the amount of DTT indicated in the text, and the diluted samples were kept on ice for 1 h before the kinase reactions were performed. Dose/response reactions for non-canalonic cyclic nucleotides were performed as described (10), using increasing concentrations of the indicated cyclic nucleotides. Cyclic nucleotide Ks values were calculated and compared using GraphPad Prism 8. Reactions were initiated by adding 10 μl diluted kinase to 5 μl 3x kinase reaction mix [120 mM Hepes (pH 7.4), 30 mM MgCl2, 180 μM ATP, 180 μCi/ml [γ-32P] ATP, and 1.56 mg/ml Kemptide] with or without 30 μM cGMP. Kinase reactions were run for 1.5 min at 30 °C and stopped by spotting on P81 phosphocellulose paper. The P81 paper was washed four times in 2 l of 0.452% metaphosphoric acid, once in 95% EtOH, and dried in an 80 °C oven. Phosphate incorporation was determined by liquid scintillation counting.

**Western blotting for purified PKGI proteins**

Purified PKGI samples were diluted ~1:100 in KPEB buffer and mixed with 2:1 with 3x SDS-loading buffer containing 300 mM maleimide. Samples were loaded onto 9% SDS-PAGE gels without heating. Separated proteins were transferred to Immobilon, blocked with 5% milk in TBS. Blots were probed with HRP-conjugated anti-Flag antibody at a 1:5000 dilution in 5% milk.

**Analysis of VASP phosphorylation in H9c2(2-1) and C2C12 cells**

H9c2(2-1) and C2C12 cells were split into 12-well cluster dishes and 24-h later, wells were treated with 8-CPT-cGMP or H2O2 as indicated in the figure legends. Cells were lysed in ice cold Buffer A containing 100 mM maleimide. Lysates were cleared by centrifugation and aliquots were added to 3x SDS sample buffer with or without β-mercaptoethanol. Reduced samples were boiled at 100 °C for 5 min before loading on 9% SDS-PAGE gels. Nonreduced samples were loaded onto the gels without boiling. Western blots were performed as described above, using the indicated antibodies.

**Data availability**

All supporting data is in the article.

---

**Supporting information**—This article contains supporting information.

**Author contributions**—S. A., T. H., and D. E. C. investigation; R. B. P. funding acquisition; R. B. P. writing–review and editing; D. E. C. conceptualization; D. E. C. methodology; D. E. C. formal analysis; D. E. C. writing–original draft; D. E. C. data curation; D. E. C. visualization; D. E. C. supervision; D. E. C. project administration.

**Funding and additional information**—This work was supported in part by National Institutes of Health Grant RO1-HL132141 (to R. B. P.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: H2O2, hydrogen peroxide; HRP, horseradish peroxidase; PKGI, Type I cGMP-dependent protein kinase; sGC, soluble guanylate cyclase; VASP, vasodilator-stimulated phosphoprotein.

**References**

1. Feil, R., Hofmann, F., and Kleppisch, T. (2005) Function of cGMP-dependent protein kinases in the nervous system. Rev. Neurosci. 16, 23–41

2. Hoffmann, L. S., Larson, C. J., and Pfeifer, A. (2016) cGMP and brown adipose tissue. Handb. Exp. Pharmacol. 233, 283–299
Oxidation activates PKGα in vitro but not in cells

cause of injury during sepsis. Proc. Natl. Acad. Sci. U. S. A. 110, 9909–9913
24. Rudyk, O., Prysyazhny, O., Burgoyne, J. R., and Eaton, P. (2012) Nitro-
glycerin fails to lower blood pressure in redox-defa Cys42Ser PKGα knock-in mouse. Circulation 126, 287–295
25. Surks, H. K., Mochizuki, N., Kasi, Y., Georgescu, S. P., Tang, K. M., Ito,
M., et al. (1999) Regulation of myosin phosphate by a specific inter-
action with cGMP-dependent protein kinase Iα. Science 286, 1583–1587
26. Kato, M., Blanton, R., Wang, G. R., Judson, T. J., Abe, Y., Myoishi,
M., et al. (2012) Direct binding and regulation of RhoA protein by cyclic
GMP-dependent protein kinase Iα. J. Biol. Chem. 287, 41342–41351
27. Tang, K. M., Wang, G. R., Lu, P., Karas, R. H., Aronovitz, M., Heximer,
S. P., et al. (2003) Regulator of G-protein signaling-2 mediates vascular
smooth muscle relaxation and blood pressure. Nat. Med. 9, 1506–1512
28. Michael, S. K., Surks, H. K., Wang, Y., Zhu, Y., Blanton, R., Jamognitj,
M., et al. (2008) High blood pressure arising from a defect in vascular
function. Proc. Natl. Acad. Sci. U. S. A. 105, 6702–6707
29. Blanton, R. M., Makimoto, E., Aronovitz, M., Thooren, R., Kass, D. A.,
Karas, R. H., et al. (2013) Mutation of the protein kinase Iα alpha leucine
zipper domain produces hypertension and progressive left ventricular
hypertrophy: a novel mouse model of age-dependent hypertensive heart
disease. J. Gerontol. A Biol. Sci. Med. Sci. 68, 1351–1355
30. Qin, L., Reger, A. S., Guo, E., Yang, M. P., Zwart, P., Casteel,
D. E., et al. (2015) Structures of cGMP-dependent protein kinase (PKG)
Iα leucine zippers reveal an interchain disulfide bond important for
stability. Biochemistry 54, 4419–4422
31. Schroder, E., and Eaton, P. (2008) Hydrogen peroxide as an endogenous
mediator and exogenous tool in cardiovascular research: issues and
considerations. Curr. Opin. Pharmacol. 8, 153–159
32. Mittal, C. K., and Murad, F. (1977) Activation of guanylate cyclase by
superoxide dismutase and hydrogen radical: a physiological regulator of
guanosine 3′,5′-monophosphate formation. Proc. Natl. Acad. Sci. U. S. A.
74, 4390–4394
33. Burke, T. M., and Wolin, M. S. (1987) Hydrogen peroxide elicits pul-
monary arterial relaxation and guanylate cyclase activation. Am. J. Physiol.
252, H721–H732
34. Wolin, M. S., and Burke, T. M. (1987) Hydrogen peroxide elicits activa-
tion of bovine pulmonary arterial soluble guanylate cyclase by a
mechanism associated with its metabolism by catalase. Biochem. Biophys.
Res. Commun. 143, 20–25
35. Brandwein, H. J., Lewicki, J. A., and Murad, F. (1981) Reversible inacti-
vation of guanylate cyclase by mixed disulfide formation. J. Biol. Chem.
256, 2958–2962
36. Humphries, K. M., Pennypacker, J. K., and Taylor, S. S. (2007) Redox
regulation of cAMP-dependent protein kinase signaling: kinase versus
phosphatase inactivation. J. Biol. Chem. 282, 22072–22079
37. Hagiwara, M., Alberts, A., Brindle, P., Meinholz, J., Feramisco, J., Deng,
T., et al. (1992) Transcriptional attenuation following cAMP induction
requires PROM1-mediated dephosphorylation of CREB. Mol. Biol. Cell.
3, 113–122
38. Wadzinski, B. E., Wheat, W. H., Jaspers, S., Peruski, L. F., Jr., Lickteig,
R. L., Johnson, G. L., et al. (1993) Nuclear protein phosphatase 2A de-
phosphorylates protein kinase A-phosphorylated CREB and regulates
CREB transcriptional stimulation. Mol. Cell. Biol. 13, 2822–2834
39. Gudi, T., Casteel, D. E., Vinson, C., Boss, G. R., and Pilz, R. B. (2000) NO
activation of fos promoter elements requires nuclear translocation of G-
kine I and CREB phosphorylation but is independent of MAP kinase
activation. Oncogene 19, 6324–6333
40. Kim, H. S., Song, M. C., Kwak, I. H., Park, T. I., and Lim, I. K. (2003)
Constitutive induction of p-Erk1/2 accompanied by reduced activities of
protein phosphatases 1 and 2A and MKP3 due to reactive oxygen species
during cellular senescence. J. Biol. Chem. 278, 37497–37510
41. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) A general method of
in vitro preparation and specific mutagenesis of DNA fragments: study of
protein and DNA interactions. Nucleic Acids Res. 16, 7351–7367
42. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989)
Site-directed mutagenesis by overlap extension using the polymerase
chain reaction. Gene 77, 51–59