Hydrogen Peroxide Enhances Vasodilatation by Increasing Dimerization of cGMP-Dependent Protein Kinase Type Iα

Dou Dou, PhD; Xiaoxu Zheng, PhD; Juan Liu, BSc; Xiaojian Xu, BSc; Liping Ye, PhD; Yuansheng Gao, PhD

Background: cGMP-dependent protein kinase type I (PKG I) plays a key role in vasodilatation caused by cGMP-elevating agents. It is a homodimer in mammalian cells, existing as 2 isoforms, Iα and Iβ. The aim of the present study was both to determine whether PKG I dimerization and activity are modulated by hydrogen peroxide (H₂O₂) and its influence on vasodilatation.

Methods and Results: The dimers and monomers of total PKG I and PKG Iβ were analyzed by Western blotting. PKG I activity was assayed by measuring the incorporation of 32P into BPDEtide. Changes in vessels tension were determined by organ chamber technique. In isolated porcine coronary arteries, H₂O₂ increased the dimers of total PKG I in a concentration-dependent manner, but had no effect on dimerization of PKG Iβ. The dimerization of PKG I caused by H₂O₂ was prevented by catalase but not by deferoxamine and tiron. H₂O₂ enhanced the activity of PKG I and relaxations of porcine coronary arteries to the nitric oxide donor and 8-Br-cGMP. Inhibition of catalase under in vivo conditions significantly decreased rat mean arterial pressure, which was associated with increased dimerization of PKG I.

Conclusions: The present study suggests that H₂O₂ may enhance the activity of PKG Iα- and PKG I-dependent vasodilatation via increased dimerization of the enzyme. (Circ J 2012; 76: 1792–1798)

Key Words: Coronary circulation; Reactive oxygen species; Vasodilation
obtained from Sigma-Aldrich, St. Louis, MO, USA); 2,3-dime-thoxy-1,4-naphthoquinone (DMNQ), 3-amino-1,2,4-triazole (3-AT); 8-Br-cGMP (Biolog Life Science Institute, Bremen, Germany), BPDEtide (Biomol Research Laboratories, Plymouth Meeting, PA, USA), deferoxamine, 2,2′-(hydroxyisotrizohydrozono) bis(ethanamine) (DETA NONOate; Cayman Chemical, Ann Arbor, MI, USA), indomethacin, nitro-L-arginine, tiron, and U46619 (9-(11,9e)-epoxymethano-prostaglandin F2α).

Indomethacin (10⁻⁵ mol/L) was prepared in equal molar Na₂CO₃, and DETA NONOate was dissolved in 0.01 mol/L NaOH. Such concentrations of Na₂CO₃ or NaOH did not significantly affect the pH of the solution in the organ chamber. The other drugs were prepared using distilled water.

**Western Blotting Analysis**

Rings of porcine coronary artery were incubated in the modified Krebs-Ringer bicarbonate buffer aerated with 95% O₂–5% CO₂ (37±0.01°C, pH=7.4) in the presence of solvent and H₂O₂ (10⁻⁵–10⁻³ mol/L) for 60 min, or in the presence of solvent and DMNQ (10⁻⁴ mol/L) for 2 h. In some experiments, rings in the solvent and H₂O₂ (10⁻³ mol/L) groups were co-incubated with catalase (2,000 U/ml), deferoxamine (DFO, 10⁻³ mol/L) or tiron (10⁻⁴ mol/L) for 60 min.

Following incubation the arteries were snap-frozen in liquid nitrogen and homogenized in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 20 mmol/L MgCl₂, 0.2 mmol/L isobutylmethylxanthine, 100 μmol/L nitro-L-arginine, and 10 μmol/L indomethacin. The homogenate was sonicated and centrifuged at 14,000 g for 10 min at 4°C. Supernatants were assayed for PKG activity by measuring the incorporation of ³²P from γ-³²P-ATP into a specific PKG substrate BPDEtide. Aliquots (20 μl) of supernatant were added to a mixture (total volume, 50 μl) containing 50 mmol/L Tris-HCl (pH 7.4), 20 mmol/L MgCl₂, 0.1 mmol/L isobutylmethylxanthine, 10 μmol/L indomethacin, 100 μmol/L nitro-L-arginine, 150 μmol/L BPDEtide, 1 μmol/L PKI (a synthetic PKA inhibitor; Peninsula Laboratories, Belmont, CA, USA), 0.2 mmol/L γ-³²P-ATP (specific activity: 3,000 Ci/mmol/L), and with or without H₂O₂ (3×10⁻⁴ mol/L). The mixture was incubated at 30°C for 10 min in the presence or absence of 3×10⁻⁵ mol/L exogenous cGMP. Reaction was terminated by spotting 40-μl aliquots of mixture onto phosphocellulose papers (2×2 cm; P81 Whatman) and placing them in ice-cold 75-mmol/L phosphoric acid. The filter papers were washed, dried, and counted in a liquid scintillation counter. Assays were performed in triplicate with appropriate controls. PKG activity is expressed as pmol of ³²P-incorporated into PKG substrate min/mg protein.

**Organ Chamber Study**

Isolated vessel rings were suspended in organ chambers by 2 stirrups passing through the lumen. One stirrup was anchored to the bottom of the organ chamber and the other was connected to a strain gauge (PowerLab/8sp, ADInstruments, Australia) for the measurement of isometric force. The organ chambers were filled with 8 ml of the modified Krebs-Ringer bicarbonate solution aerated with 95% O₂–5% CO₂ maintained at 37±0.1°C with pH=7.4.

At the beginning of the experiment, each coronary artery vessel ring was stretched to its optimal resting tension, which was ~2.5 g. This was achieved by step-wise stretching in 0.5-g increments. After the vessels were brought to their optimal resting tension, 1 h of equilibration was allowed.

Relaxation of isolated porcine coronary artery in response to DETA NONOate (a stable NO donor) or 8-Br-cGMP was determined in vessels pre-constricted with U46619 (3×10⁻⁷ mol/L) to a similar level of tension. Effects of H₂O₂ (3×10⁻⁴ mol/L) on the vasodilator response of the vessels were examined 60 min after administration. The concentration-response curves to the vasodilators were constructed in a cumulative fashion. Each concentration was added only when the response to the prior one became stable. In all experiments indomethacin (10⁻⁵ mol/L) and nitro-L-arginine (10⁻⁴ mol/L) were added at least 30 min before vessels were constricted with U46619 and remained in contact with the tissue throughout the experiment. All experiments were carried out in a parallel fashion under control conditions or with different treatments.

**Measurement of Rat Mean Arterial Pressure**

Male Sprague-Dawley rats (220–230 g body weight) were used. After they were lightly anaesthetized with intraperitoneal injection of pentobarbital sodium (30 mg/kg), the carotid artery and the contralateral jugular vein were cannulated. The cannula of the carotid artery was connected to a transducer of PowerLab/
8sp (ADInstruments, Australia) for measuring the rat's blood pressure, including systolic and diastolic pressures and mean arterial pressure. The cannula of the jugular vein was used to infuse saline or 3-amino-1,2,4-triazole (3-AT, 0.1 g/ml). When the blood pressure were stable after infusion of saline or 3-AT for 30 min, the rat was killed and its aortas quickly dissected out and frozen in liquid nitrogen. The protein levels of PKG dimers and monomers were assayed by Western blotting as described earlier.

The study protocol and the use of rats were reviewed and approved by the Animal Care and Use Review Committees of Peking University Health Science Center. The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Data Analyses
Data are shown as mean±SEM. When mean values of 2 groups were compared, Student’s t-test for unpaired observations was used. When the mean values of the same group before and after stimulation were compared, Student’s t-test for paired observations was used. Comparison of mean values of more than 2 groups was performed with 1-way ANOVA test with Student-Newman-Keuls test for post hoc testing of multiple comparisons. Statistical significance was accepted when the P value (2-tailed) was less than 0.05. In all experiments, n represents the number of animals.

Results
PKG I Dimerization
H$_2$O$_2$ significantly increased the protein level of the total PKG I dimers of porcine coronary arteries in a concentration-dependent manner, but the monomers of PKG I were not affected (Figure 1A). H$_2$O$_2$ (from 10$^{-5}$ mol/L to 10$^{-3}$ mol/L) had no effect on the dimeric status of PKG I$\beta$ (Figure 1B).

The increase in PKG I dimers caused by H$_2$O$_2$ (10$^{-3}$ mol/L) was prevented by catalase (2,000 U/ml), but was not affected by DFO (10$^{-4}$ mol/L) or tiron (10$^{-2}$ mol/L) (Figure 2).

DMNQ (10$^{-4}$ mol/L), an endogenous reactive oxygen species (ROS) generator, also increased the dimerization of PKG I of porcine coronary arteries, but had no effect on PKG I monomers (Figure 3). The effect of H$_2$O$_2$ (10$^{-3}$ mol/L) on PKG dimerization was not affected by a protein synthesis inhibitor (cycloheximide, 10$^{-4}$ mol/L) or a protein degradation inhibitor (MG-132, 10$^{-5}$ mol/L) (data not shown, n=5).

Expression of PKG I in Cytoplasm and Membrane
Incubation of porcine coronary arteries with H$_2$O$_2$ (10$^{-3}$ mol/L) for 1 h increased the level of PKG I in membrane significantly, but did not affect that in cytoplasm (Figure 4).

PKG Activity Assay
H$_2$O$_2$ (3×10$^{-4}$ mol/L) had no effect on the basal activity of PKG I in the porcine coronary arteries but significantly increased the activity stimulated by cGMP (3×10$^{-4}$ mol/L; Figure 5).

Organ Chamber Study
Relaxant responses of porcine coronary artery were determined in vessels constricted to similar tension levels with U46619 (1–2×10$^{-7}$ mol/L, tension ranged from 13.9±1.6 to 16.4±1.3 g, n=5–8, P>0.05) and in the presence of indomethacin (10$^{-5}$ mol/L) and nitro-L-arginine (10$^{-4}$ mol/L) to prevent possible interference of endogenous cyclooxygenase prod-
In our preliminary experiments, we determined that H$_2$O$_2$ (3×10^{-4} mol/L) has no significant effect on the constriction of porcine coronary arteries caused by U46619 at 10^{-7} mol/L (11.3±2.0 g vs. 12.5±2.6 g, n=6, P>0.05).

DETA NONOate (a stable NO donor) caused a concentration-dependent relaxation of porcine coronary artery. The effect was inhibited by Rp-8-Br-PET-cGMPS (3×10^{-5} mol/L),
a specific inhibitor of PKG. Relaxation caused by DETA NONOate was significantly enhanced by H$_2$O$_2$ ($3\times10^{-4}\text{mol/L}$) and this effect was largely inhibited by Rp-8-Br-PET-cGMPS ($3\times10^{-5}\text{mol/L}$). Data are shown as mean±SEM; n=5-8 for each group. *Significantly different between control and H$_2$O$_2$ groups (P<0.05); †significantly different from vessels treated with Rp-8-Br-PET-cGMPS (P<0.05). (Figure 7).

Effects of 3-AT on Rat Mean Arterial Pressure and PKG I Dimers of the Aorta

There were no differences in the basal mean arterial pressure of rats in the saline group and the 3-AT group (133.7±4.4 mmHg vs. 135.3±5.3 mmHg, n=4-6, P>0.05). Intravenous injection of 3-AT (0.2 g/100 g body weight) in the jugular vein significantly decreased the mean arterial pressure of the rat (A), which was associated with an increased dimerization of PKG I and a decreased level of PKG I monomers (B). Data are shown as mean±SEM; n=4–6 for each group. *Significantly different from the basal mean arterial pressure in 3-AT group (P<0.05) (A); †significant difference in the level of PKG I monomers between saline and 3-AT groups (P<0.05) (B); †significant difference in the level of PKG I dimers between saline and 3-AT groups (P<0.05). 3-AT, 3-amino-1,2,4-triazole; PKG I, cGMP-dependent protein kinase type I.
cantly decreased the mean arterial pressure of rats more than the administration of saline (Figure 8A). Intravenous injection of 3-AT (0.2 g/100 g body weight) significantly increased the protein level of PKG I dimers, while decreasing that of PKG I monomers (Figure 8B).

**Discussion**

PKG I is highly expressed in vascular smooth muscle cells and plays a pivotal role in mediating vasodilatation caused by cGMP-elevating agents, including endothelium-derived NO, natriuretic peptides, and nitrosavulators such as nitroglycerin.\(^1\)\(^-\)\(^3\)\(^-\)\(^11\) PKG I is a homodimer. The dimeric status enhances its catalytic activity and thereby its biological actions.\(^5\)

In the present study we demonstrated that the dimerization of PKG I is enhanced by H\(_2\)O\(_2\), associated with increased activity of the enzyme and augmented relaxant responses to a NO donor and an analog of cGMP. Moreover, the enhancement effects of H\(_2\)O\(_2\) were prevented by inhibition of PKG I with Rp-8-Br-PET-cGMPS. These results suggest that augmented vasodilatation caused by the peroxide may result from increased PKG I dimerization. These observations are in line with findings reported recently in bovine pulmonary arteries, rat isolated heart, and human coronary arteries.\(^6\)\(^-\)\(^8\)

PKG I has 2 isoforms, \(\alpha\) and \(\beta\). They differ in the first 90–100 residues of the N terminus. Both isoforms are involved in mediating vasodilatation induced by cGMP, with \(\alpha\) being approximately 10-fold more sensitive to cGMP.\(^1\)\(^-\)\(^3\) In the present study the protein levels of PKG I dimers determined using an antibody that does not distinguish the I\(_\alpha\) from I\(_\beta\) isoform were increased by H\(_2\)O\(_2\). However, the protein levels of PKG I\(_\alpha\) dimers determined using antibody specifically against PKG I\(_\beta\) were not affected by H\(_2\)O\(_2\), which suggests that H\(_2\)O\(_2\) may affect PKG I\(_\alpha\) specifically. It is postulated that PKG I\(_\alpha\) forms a homodimers via a interchain disulfide between Cys42 residues on adjacent chains.\(^6\)

ROS, including superoxide anion, H\(_2\)O\(_2\) and hydroxyl radical, are generated as intermediates in reduction-oxidation reactions.\(^12\) H\(_2\)O\(_2\) is primarily transformed from superoxide anion by superoxide dismutase, and can be catalyzed to water by catalase or converted to hydroxyl radical in the presence of transition ions such as Fe\(^2+\).\(^13\)\(^-\)\(^14\) In this study, the dimerization of PKG I was not affected by iron, a scavenger of superoxide anion,\(^15\) or by deferoxamine, which prevents the formation of hydroxyl radical by chelating Fe\(^2+\) in the cells.\(^16\) The increase in PKG I dimers induced by H\(_2\)O\(_2\) was abolished by catalase, suggesting that H\(_2\)O\(_2\) is the particular ROS species that modulates the dimerization of PKG I\(_\alpha\).

DMNQ disrupts the mitochondrial respiratory chain and increases production of endogenous ROS.\(^17\) In the present study, DMNQ significantly increased the level of PKG I dimers in porcine coronary arteries. Because DMNQ can increase the intracellular production of H\(_2\)O\(_2\), and PKG I dimerization caused by exogenous H\(_2\)O\(_2\) is abolished by catalase but not affected by scavengers of superoxide and hydroxyl radicals, it is likely that an increased intracellular H\(_2\)O\(_2\) level promotes PKG I dimerization. These results suggest that not only exogenous but also endogenous H\(_2\)O\(_2\) could augment the dimers of PKG I. H\(_2\)O\(_2\) is implicated in the regulation of vascular reactivity under physiological and pathophysiological conditions.\(^18\)\(^-\)\(^20\) Its action via PKG I dimerization may exert a protective effect on vascular functions.

PKG I exists predominately in the cytosol. Many substrates of PKG I are located in the membranes of cells such as the large conductance calcium-activated potassium channel (BK channel).\(^8\) In our study, 1 h incubation with H\(_2\)O\(_2\) (10\(^{-3}\) mol/L) significantly increased the level of PKG I in the membrane but not the cytosol preparations. To determine whether the increased PKG I dimers induced by H\(_2\)O\(_2\) were affected by inhibition of protein synthesis and degradation, we treated porcine coronary arteries with a protein synthesis inhibitor (cycloheximide) and a protein degradation inhibitor (MG-132) and found that they did not affect the augmentation of PKG I dimerization caused by H\(_2\)O\(_2\). We also found the inhibition of catalase by 3-AT under in vivo conditions potentiated the level of PKG I dimers while attenuating that of PKG I monomers in rat aortas, suggesting that H\(_2\)O\(_2\) promotes the transmormation of PKG I from monomers to dimers. In our study using porcine coronary arteries, H\(_2\)O\(_2\) increased the level of PKG I dimers but had no significant effect on the protein level of monomers. H\(_2\)O\(_2\) augmented the total PKG I in the cell membrane but not that in the cytosol. The difference between rat aortas and porcine coronary arteries is unclear. We suspect that in porcine coronary smooth muscle cells PKG I is predominantly cytosolic, so the amount of PKG I translocated to the membrane by H\(_2\)O\(_2\) would not appreciably affect the total PKG I level in the cytoplasm. A recent study suggests that H\(_2\)O\(_2\) may increase the activity of BK channels through PKG I dimerization and translocation to the plasma membrane.\(^8\)

The dimerization of PKG I increases its sensitivity to cGMP activation.\(^3\) In our study, nitro-L-arginine and indomethacin were included in the buffer of PKG activity assay to avoid production of endogenous cGMP and cAMP by inhibition of NO synthase and cyclooxygenase. Therefore, although H\(_2\)O\(_2\) could increase PKG Ia dimerization, there was little enzyme substrate cGMP, so there was no difference between the control and H\(_2\)O\(_2\) groups in the activity of PKG Ia. When stimulated with exogenous cGMP, H\(_2\)O\(_2\) augmented PKG Ia activity and relaxation of the vessels induced by NO donor or 8-Br-cGMP. It suggests that H\(_2\)O\(_2\) acts directly on the target of cGMP (ie, PKG). Such a postulation is in line with observations that the effects of NO and 8-Br-cGMP were abolished by the inhibitor of PKG. A recent study suggests that, in human coronary arterioles, the effect of H\(_2\)O\(_2\) on Ca\(^{2+}\)-activated potassium channels is primarily through PKG.\(^8\) As a potent thiol oxidant, H\(_2\)O\(_2\) may affect the activities of many enzymes via its dimerization effect. Whether or not H\(_2\)O\(_2\) modulates PKG action through the dimerization of the distal target of PKG remains to be explored.

In the coronary arteries, H\(_2\)O\(_2\) is generated locally and may act as an endothelium-derived hyperpolarizing factor.\(^18\) In a number of vessel types, H\(_2\)O\(_2\) can directly activate Ca\(^{2+}\)-activated potassium channels and thereby lead to membrane hyperpolarization and vasodilatation.\(^18\)\(^-\)\(^21\)\(^-\)\(^24\) In addition to acting as a mediator of dilation, our present study and recent studies by others suggest that H\(_2\)O\(_2\) may also act as a facilitator of dilation by enhancing the activity of PKG Ia via dimerization.\(^6\)\(^-\)\(^8\)

In the present study, PKG I dimerization was affected when treatment was with H\(_2\)O\(_2\) at 10\(^{-3}\) mol/L or more. These results are in line with published data.\(^6\)\(^-\)\(^7\) The effective concentration of H\(_2\)O\(_2\) that affects the dimeric status of PKG I is relatively high, indicating that this mechanism may operate under pathophysiological conditions such as hypoxia, ischemia-reflow injury and inflammation.\(^18\)\(^-\)\(^25\)\(^-\)\(^26\) 3-AT is an irreversible inhibitor of catalase\(^27\) that has been used to elevate the in vivo H\(_2\)O\(_2\) concentration.\(^28\)\(^-\)\(^29\) In present study, we found that intravenous injection of 3-AT significantly decreased the mean arterial pressure of rats, which was associated with increased dimer-
ization of PKG I. These results suggest that the mechanism by which H$_2$O$_2$ enhances the activity of PKG I and PKG I-dependent relaxation via increased dimerization of the enzyme is operative under in vivo conditions. H$_2$O$_2$-mediated PKG I dimerization may also operate under physiological conditions. There are studies suggesting that 300 micromolar H$_2$O$_2$ is a physiological concentration achieved in the vasculature.\cite{10, 19, 20} The NO-cGMP-PKG signaling pathway is critically important in the regulation of vascular reactivity.\cite{11, 4, 5, 6}

The present study and other recent studies\cite{8, 9} suggest that H$_2$O$_2$-mediated redox modulation of PKG and probably its downstream effectors may be an important mechanism in the regulation of vascular reactivity.

**Acknowledgments**

This work was supported by the National Natural Science Foundation of China (Grant No. 50870938 and 81001433), Doctoral Fund of Ministry of Education for New Teachers (Grant No. 20100001120037) and Peking University Health Science Center Foundation for New Teachers (Grant No. BMU20090510).

**References**