Gene Transfer of cGMP-Dependent Protein Kinase I Enhances the Antihypertrophic Effects of Nitric Oxide in Cardiomyocytes

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Abstract—NO acting through soluble guanylyl cyclase and cGMP formation is a negative regulator of cardiomyocyte hypertrophy. Downstream targets mediating the inhibitory effects of NO/cGMP on cardiomyocyte hypertrophy have not been elucidated. In addition to its antihypertrophic effects, NO promotes apoptosis in cardiomyocytes, presumably through cGMP-independent pathways. We investigated the role of cGMP-dependent protein kinase (PKG) in the antihypertrophic and proapoptotic effects of NO. Incubation of neonatal rat cardiomyocytes with the NO donor S-nitroso-N-acetyl-l-d,l-penicillamine (SNAP) (250 μmol/L) or the PKG-selective cGMP analog 8-pCPT–cGMP (500 μmol/L) activated endogenous PKG type I, as shown by the site-specific phosphorylation of vasodilator-stimulated phosphoprotein, a well-characterized PKG substrate. SNAP (250 μmol/L) and 8-pCPT–cGMP (500 μmol/L) modestly attenuated the hypertrophic response to α1-adrenergic stimulation with phenylephrine. Although a high concentration of SNAP (1000 μmol/L) promoted apoptosis in cardiomyocytes, as evidenced by the formation of histone-associated DNA fragments, antihypertrophic concentrations of SNAP (250 μmol/L) and 8-pCPT–cGMP (500 μmol/L) did not promote cell death. Because chronic activation downregulated endogenous PKG I, we explored whether gene transfer of PKG I would enhance the sensitivity of cardiomyocytes to the antihypertrophic effects of NO/cGMP. Indeed, after adenoviral overexpression of PKG I, SNAP (250 μmol/L) and 8-pCPT–cGMP (500 μmol/L) completely suppressed the hypertrophic response to α1-adrenergic stimulation. As observed in noninfected cells, SNAP (250 μmol/L) and 8-pCPT–cGMP (500 μmol/L) did not promote apoptosis in cardiomyocytes overexpressing PKG I. Moreover, overexpression of PKG I did not enhance the proapoptotic effects of 1000 μmol/L SNAP, implying PKG-independent effects of NO on apoptosis. Endogenous PKG I mediates antihypertrophic but not proapoptotic effects of NO in a cell culture model of cardiomyocyte hypertrophy. Adenoviral gene transfer of PKG I selectively enhances the antihypertrophic effects of NO without increasing the susceptibility to apoptosis. (Hypertension. 2002;39:87-92.)

Key Words: nitric oxide ■ cyclic GMP ■ protein kinases ■ hypertrophy ■ apoptosis

Cardiac hypertrophy is a compensatory mechanism of the heart, as it is critical for the maintenance of normal contractile function in response to chronic increases in hemodynamic load. Hypertrophy can initially be viewed as a salutary response; ultimately, however, it often heralds decompensation and transition to heart failure.1

Whereas the signal transduction pathways promoting cardiomyocyte hypertrophy have been characterized in great detail, there is insufficient knowledge regarding signaling pathways that oppose cardiomyocyte hypertrophy. It has been reported, however, that NO, through activation of soluble guanylyl cyclase and cGMP formation, attenuates the hypertrophic response to growth factor stimulation in cardiomyocytes.2,3 In agreement with these in vitro studies, treatment of spontaneously hypertensive rats with the NO precursor l-arginine increases cardiac NOX and cGMP content and inhibits cardiac hypertrophy independent of blood pressure.4 In addition to its antihypertrophic effect, NO has been shown to promote apoptosis in cardiomyocytes in a dose-dependent manner.5,6 However, the role of cGMP in the proapoptotic effects of NO is controversial. In fact, cGMP analogs have been reported both to induce5 and not to induce6 apoptosis in cardiomyocytes, and it has been proposed that NO promotes cardiomyocyte apoptosis by cGMP-independent mechanisms through formation of peroxynitrite.6–8

Downstream targets for cGMP-mediated antihypertrophic and, possibly, proapoptotic effects in cardiomyocytes have not been elucidated. In general, cGMP effectors include
cGMP-regulated phosphodiesterases, cGMP-regulated ion channels, and cGMP-dependent protein kinases (PKG). Two PKG genes have been identified in mammalian cells, encoding for PKG type I (including α- and β-splice variants) and PKG type II. In cardiomyocytes, PKG I has been suggested to mediate negative inotropic effects of NO/cGMP, possibly through regulation of the L-type Ca\(^{2+}\) channel and troponin I, thereby reducing Ca\(^{2+}\)-influx and myofilament Ca\(^{2+}\) sensitivity. However, a role for PKG I in controlling cardiomyocyte hypertrophy and/or apoptosis has not been reported. In this study, we demonstrate for the first time that PKG I mediates antihypertrophic but not proapoptotic effects of NO in cardiomyocytes.

Methods

Materials

8-Para-chlorophenylthio–cGMP (8-pCPT–cGMP) and Sp-5,6-DCI-cBIMPS (cBIMPS) were purchased from Biolog. S-nitroso-N-acetyl-d,l-penicillamine (SNAP) and phenylephrine (PE) were obtained from Calbiochem and Sigma, respectively.

Cardiomyocyte Culture

Ventricular cardiomyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats by Percoll gradient. Cells were plated overnight in Dulbecco’s modified Eagle’s medium (DMEM)/medium-199 (4:1) supplemented with 10% horse serum, 5% fetal calf serum, glutamine, and antibiotics. The next morning, cardiomyocytes were switched to DMEM/medium-199 supplemented only with glutamine and antibiotics (maintenance medium).

Immunofluorescence and Immunoblotting

Cardiomyocytes were immunostained for troponin T and PKG I according to a previously published procedure, with a monoclonal antibody directed against troponin T (Sigma) and a polyclonal antibody directed against PKG I (C). To determine cell viability, cardiomyocytes were double-labeled with antibodies against troponin T (A) and PKG I (B). Troponin T labeling identified the PKG I-containing cells as cardiomyocytes (magnification, ×400). PKG I expression was most abundant at sites of cell-cell contact (arrows in B). Homogenates (30 μg protein/lane) from purified ventricular cardiomyocytes (CM) or 1-day-old rat heart ventricles were analyzed by immunoblotting with specific polyclonal antibodies against PKG I (C) or PKG II (D). At left, recombinant PKG I and II standards (Std) were used as migration markers.

Adenoviral Infection of Cardiomyocytes

A replication-deficient adenovirus containing the cDNA of human PKG Iβ (Ad.PKG Iβ)17 was used to overexpress PKG I in cardiomyocytes. As a control, we used a replication-deficient adenovirus encoding a catalytically inactive form of PKG Iβ (Ad.PKG Iβ–K405A), generated by replacing Lys405 in the ATP-binding site of PKG Iβ by Ala. After overnight plating, cardiomyocytes were switched to maintenance medium and infected for 2 hours with recombinant adenoviruses. After removal of the virus suspension, cardiomyocytes were incubated in maintenance medium supplemented with 5% fetal calf serum to allow for expression of wild-type or mutated PKG Iβ. After 24 hours, cardiomyocytes were switched back to maintenance medium and stimulated with various agents. Cardiomyocytes were routinely infected with 1×10¹⁷ viral particles/cell. At this concentration, Ad.PKG Iβ augmented cGMP-stimulated PKG activity (determined by in vitro kinase assay19) 12.9-fold, as compared with control (Ad.PKG Iβ infected cells: 4.39±0.35 nmol/min per milligram of protein; Ad.PKG Iβ–K405A-infected cells: 0.28±0.02 nmol/min per milligram of protein; noninfected control cells: 0.34±0.06 nmol/min per milligram of protein).

Assessment of Cardiomyocyte Hypertrophy and Survival

Cardiomyocyte surface area was determined by planimetry with phase contrast microscopy and a digital image analyzer (Leica Q50 MC). [3 H]leucine incorporation was measured as an index of total protein synthesis.20 Prepro ANP mRNA/18S expression was assessed by Northern blot hybridization. To determine cell viability, cells were analyzed by phase contrast microscopy immediately before the 24-hour stimulation period. For each condition, a random field (~200 cells) was chosen and marked on the outside of the well. Cells attached to the gelatin matrix were counted. After 24 hours, the same field was located, and cell survival was determined by trypan blue exclusion. The formation of histone-associated DNA fragments was quantified by cell death detection ELISAPLUS (Roche). As a positive control for apoptosis, cardiomyocytes were subjected to serum and glucose deprivation.

Statistical Analysis

Data are presented as mean±SEM. Results are from ≥3 (where shown) experiments. Differences between groups were analyzed by 1-way ANOVA followed by Student’s t test with Bonferroni correction. A 2-tailed value of P<0.05 was considered to indicate statistical significance.

Results

Cardiomyocytes Express Functionally Active Endogenous PKG I

PKG type I was readily detectable in neonatal cardiomyocytes by immunofluorescence (Figure 1, A and B) and by immunoblotting (Figure 1C), whereas PKG type II was undetectable (Figure 1D). PKG I expression was most abundant at sites of cell-cell contact (arrows in panel B). Expression levels of PKG I in ventricular cardiomyocytes and in 1-day-old rat heart ventricles were comparable, indicating that myocytes significantly contribute to the expression of PKG I in neonatal heart (Figure 1C). To demonstrate functional activity of endogenous PKG I in cardiomyocytes, we analyzed the phosphorylation status of VASP, a cytoskeleton-associated protein, and established PKG substrate in many
cell types. Stimulation of cardiomyocytes with the PKG-selective cGMP analog 8-pCPT–cGMP23 promoted phosphorylation of VASP on Ser239 (Figure 2B) and slightly on Ser157 (shift in apparent molecular weight from 46 to 50 kDa). The 16C2 monoclonal antibody, made against the VASP phosphorylation site containing Ser239,16 was used to detect Ser239 phosphorylation of VASP (B and C). At left, VASP standard (Std) from human platelets phosphorylated on both Ser157 and Ser239 is shown (A through C).

Downregulation of Endogenous PKG I Expression and Function in Response to Long-Term Activation

As shown in Figure 3, prolonged activation resulted in downregulation of endogenous PKG I expression and function in cardiomyocytes. After stimulation of cardiomyocytes with 8-pCPT–cGMP for 12 hours, PKG I expression and Ser157-phosphorylation of VASP were already reduced (Figure 3A and 3B). After 48 hours of stimulation, PKG I expression was further reduced and phosphorylation of VASP at both Ser157 and Ser239 was impaired (Figure 3A through 3C).

Gene Transfer of PKG Iβ Enhances the Inhibitory Effects of SNAP and 8-pCPT–cGMP on α1-Adrenergic Cardiomyocyte Hypertrophy

SNAP (250 μmol/L) and 8-pCPT–cGMP (500 μmol/L) partially inhibited the hypertrophic response to phenylephrine stimulation in noninfected cardiomyocytes (reduction of cell surface area and [3 H]leucine incorporation, no effect on prepro ANP mRNA expression), indicating that NO or direct stimulation of endogenous PKG I promote a modest antihypertrophic effect (Figure 4A through 4C, left panels). Because endogenous PKG I was susceptible to downregulation by prolonged stimulation, we studied whether the antihypertrophic effects of SNAP and 8-pCPT–cGMP could be enhanced by adenoviral gene transfer of PKG I. As observed in noninfected cardiomyocytes, SNAP and 8-pCPT–cGMP partially suppressed α1-adrenergic hypertrophy in cardiomyocytes infected with control viruses carrying either no foreign cDNA insert (data not shown) or expressing catalytically inactive PKG Iβ–K405A (Figure 4A through 4C, middle panels). By contrast, overexpression of wild-type PKG Iβ strongly augmented the antihypertrophic effects of SNAP and 8-pCPT–cGMP (Figure 4A through 4C, right panels). In fact, in cardiomyocytes overexpressing wild-type PKG Iβ, SNAP and 8-pCPT–cGMP completely suppressed the hypertrophic response to α1-adrenergic stimulation (cell surface area, [3 H]leucine incorporation, and ANP expression). SNAP or 8-pCPT–cGMP stimulation of either endogenous or overexpressed PKG I exerted no significant effects on basal cell area, [3 H]leucine incorporation, and ANP expression (Figure 4A through 4C).

Gene Transfer of PKG Iβ Does Not Promote Cardiomyocyte Apoptosis

Because NO donors and, in some studies, cGMP analogs have been reported to induce apoptosis in neonatal cardiomyocytes, we determined whether PKG I affects cardiomyocyte survival. Exposure of noninfected cardiomyocytes to 250 μmol/L SNAP or 500 μmol/L 8-pCPT–cGMP, that is, concentrations that partially suppressed α1-adrenergic hyper-
trophy, did not reduce cell viability and did not promote DNA fragmentation, a marker of apoptotic cell death (Figure 5A and 5B, left panels). By contrast, a higher concentration of SNAP (1000 μmol/L) resulted in a significant decrease in cell viability accompanied by the accumulation of histone–H3 and histone–H4 (Figure 5A and 5B, right panels). SNAP and 8-pCPT–cGMP exerted similar effects on cardiomyocyte survival in the presence or absence of phenylephrine (Figure 5A and 5B).

Discussion

This study provides the first evidence that cGMP-dependent protein kinase type I promotes antihypertrophic but not proapoptotic effects of NO/cGMP in cardiomyocytes. Furthermore, our results indicate that adenoviral gene transfer of PKG I is a powerful molecular approach to selectively potentiate the antihypertrophic effects of NO/cGMP without increasing the susceptibility to apoptotic cell death.

Consistent with previous reports that indicated that negative inotropic effects of NO/cGMP in mammalian cardiomyocytes are mediated through stimulation of PKG,10–13 our data show that neonatal rat ventricular cardiomyocytes express endogenous PKG I. This endogenous PKG I is a downstream target for NO/cGMP in cardiomyocytes, as demonstrated by the site-specific phosphorylation of VASP, a well-characterized PKG substrate,16 in response to stimulation with the NO donor SNAP or the PKG-selective cGMP analog 8-pCPT–cGMP.23 Treatment with SNAP or direct activation of endogenous PKG I with 8-pCPT–cGMP attenuated the hypertrophic response to α1-adrenergic stimulation. Inhibitory effects of NO and endogenous PKG I on α1-adrenergic hypertrophy were rather weak, however.

Similar to observations in vascular smooth muscle cells,24 prolonged activation resulted in downregulation of endogenous PKG I expression and function in cardiomyocytes. Therefore, we explored whether gene transfer of PKG I might enhance the antihypertrophic effects of NO/cGMP. Indeed, after adenoviral overexpression of PKG Iβ, SNAP and 8-pCPT–cGMP completely suppressed α1-adrenergic hypertrophy. Importantly, gene transfer of catalytically inactive PKG Iβ–K405A, carrying a point mutation in the ATP-binding site,18 did not augment the antihypertrophic effects of SNAP and 8-pCPT–cGMP. Therefore, nonspecific effects possibly related to the overexpression of a foreign protein are extremely unlikely. While our experiments were in progress, it was reported that rabbit cardiomyocytes predominantly express the α splice variant of PKG I.25 PKG Iα and PKG Iβ have been shown to bind to and phosphorylate common target proteins in heart13 but also distinct target proteins in other tissues.26 Using a replication-deficient adenovirus expressing human PKG Iα, we found that the inhibitory effects of PKG Iα overexpression on α1-adrenergic hypertrophy are virtually indistinguishable from the effects observed after gene transfer of PKG Iβ (data not shown), indicating that phosphorylation targets critical for antihypertrophic effects in heart are shared by PKG Iα and PKG Iβ.

Potential targets for PKG I in cardiomyocytes include Ca2+–dependent signaling pathways, RhoA, and, as shown here, VASP. The function of VASP in cardiomyocytes is still poorly understood. Localization of VASP at intercalated discs in cardiomyocytes27 suggests that VASP may be involved in PKG I regulation of electrical coupling.28 Moreover, because VASP has been shown to control cytoskeletal actin filament assembly in other cell types,29 VASP may play a role in mediating the effects of PKG I on cardiomyocyte surface area. Ca2+–dependent signaling pathways, such as calcineurin and Ca2+/calmodulin–dependent kinases, are crucial regulators of the hypertrophic response in cardiomyocytes.30,31 PKG I regulates intracellular [Ca2+] at multiple levels, including...
the L-type Ca$^{2+}$ channel and the 1,4,5-trisphosphate-receptor.$^{10,32}$ PKG I inhibition of the L-type Ca$^{2+}$ current, for example, may mediate negative inotropic effects of NO/cGMP in cardiomyocytes. Importantly, Ca$^{2+}$ influx through the L-type Ca$^{2+}$-channel has also been implicated in the regulation of cardiomyocyte hypertrophy.$^{33}$ Therefore, anti-hypertrophic effects of PKG I may be mediated in part through inhibition of Ca$^{2+}$-dependent signaling pathways in cardiomyocytes. The low-molecular-weight GTPase RhoA, which is required for $\alpha_1$-adrenoceptor signaling in cardiomyocytes,$^{34}$ may represent an additional PKG I target. PKG I has recently been shown to phosphorylate RhoA and inhibit its biological activity in vascular smooth muscle cells,$^{35}$ suggesting that inhibition of RhoA may contribute to the antihypertrophic effects of PKG I. Clearly, further studies will be required to identify which of its many molecular targets PKG I uses to inhibit cardiomyocyte hypertrophy.

Importantly, the concentration of SNAP that partially suppressed $\alpha_1$-adrenergic hypertrophy in noninfected cardiomyocytes did not reduce cell viability. Only exposure of cardiomyocytes to a higher concentration of SNAP induced cell death and DNA fragmentation. Direct activation of endogenous or overexpressed PKG I by 8-pCPT-cGMP did not promote apoptotic cell death. Furthermore, overexpression of PKG I$\beta$ did not enhance the susceptibility of cardiomyocytes to the proapoptotic effects of SNAP. Taken together, our data argue against a role for PKG I in promoting apoptosis in neonatal cardiomyocytes and support the concept that proapoptotic effects of NO in neonatal cardiomyocytes are mediated by cGMP-independent$^{6-8}$ rather than cGMP-dependent$^3$ mechanisms. This conclusion is further supported by immunoblotting experiments (data not shown) demonstrating that antihypertrophic concentrations of SNAP or 8-pCPT-cGMP do not promote cleavage of the caspase-3 substrate PKC$\delta$ (method according to Reference 22) in noninfected or PKG I$\beta$ overexpressing cardiomyocytes, whereas the positive control of serum and glucose deprivation always caused PKC$\delta$ cleavage. Although our data indicate that PKG I does not promote apoptosis in cardiomyocytes, we cannot rule out small effects on cardiomyocyte survival that are below the detection limit of our assays.

In summary, antihypertrophic effects of PKG I are not associated with a decrease in cardiomyocyte survival, and gene transfer of PKG I may therefore represent a valuable strategy to selectively inhibit cardiomyocyte hypertrophy. However, our data were derived from neonatal cardiomyocytes stimulated with phenylephrine in vitro, which is a limited model of cardiac hypertrophy. Therefore, in the future, studies of the signaling pathways mediating antihypertrophic effects of NO/cGMP need to be extended to investigations with adult cardiomyocytes and pressure-overload hypertrophy in vivo. In this regard, a number of recent studies suggest that NO/cGMP and ANP/cGMP signaling pathways do exert antihypertrophic effects in vivo, independent of blood pressure.$^{4,36,37}$ To characterize downstream targets mediating the antihypertrophic effects of cGMP in vivo and to further explore the role of PKG I in the adult and in vivo situation, we are currently generating transgenic mice with cardiac-specific overexpression of PKG I.

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