Nitric oxide and cGMP protein kinase activity in aged ventricular myocytes

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Received 17 May 2001; accepted in final form 24 August 2001

Zhang, Qihang, Bruno Molino, Lin Yan, Todd Haim, Yakir Vaks, Peter M. Scholz, and Harvey R. Weiss. Nitric oxide and cGMP protein kinase activity in aged ventricular myocytes. Am J Physiol Heart Circ Physiol 281: H2304–H2309, 2001.—We tested the hypothesis that nitric oxide-induced negative functional effects through cGMP would be reduced in aged cardiac myocytes. Maximum rate of shortening (R_max) and percent shortening of ventricular myocytes from young (6 mo) and old (3 y) rabbits were studied using a video edge detector. cGMP-dependent phosphorylation was examined by electrophoresis and autoradiography. Myocytes received a nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP, 10^{-7}, 10^{-6}, and 10^{-5} M) followed by KT-5823 (10^{-6} M), a cGMP protein kinase inhibitor. Baseline function was similar in young and old myocytes (89.1 ± 4.5 young vs. 86.4 ± 8.3 μm/s old R_max: 5.6 ± 0.3 vs. 5.2 ± 0.7% shortening). SNAP (10^{-5} M) decreased R_max in both young (25%, n = 6) and old myocytes (24%, n = 7). SNAP also reduced percent shortening by 28% in young and 23% in old myocytes. The negative effects of SNAP were partially reversed by KT-5823 only in young myocytes. Multiple proteins were phosphorylated by cGMP, and KT-5823 could reduce this effect. The degree of phosphorylation was significantly less in old myocytes. These results suggest that the functional response of ventricular myocytes to nitric oxide was preserved during aging. However, the importance of cGMP-dependent protein phosphorylation was decreased, indicating a shift to other pathways.

cardiac myocytes; aging; rabbit; myocyte function

Nitric oxide and its second messenger cGMP have negative metabolic and functional effects in cardiac myocytes (4, 10, 14, 21). It causes reductions in local myocardial shortening, force development, and O_2 consumption (4, 11, 18). These effects operate through protein phosphorylation by cGMP-dependent protein kinase, cGMP-regulated cAMP phosphodiesterases, and direct inhibition of L-type calcium channels (18, 21, 26, 31). Previous work demonstrated that the cGMP-dependent protein kinase was a major pathway to mediate the negative metabolic and functional effects of cGMP (21, 26, 28). Nitric oxide-dependent adaptive mechanisms are important for the prevention and treatment of cardiovascular diseases (12, 27). Synthesis and release of nitric oxide declines with age in endothelial cells, which has been implicated in the development of several age-dependent cardiovascular diseases (1, 3, 29). However, there may be increased guanylyl cyclase activity in aged cardiac myocytes (25). It was also reported that the insulin-like growth factor-1 (IGF-1)-induced cardiac contractile responses were reduced during aging, and it was suggested that increased effects of nitric oxide might be responsible (23, 24). The relative importance of the cGMP protein kinase and cGMP-dependent cAMP phosphodiesterases vary under different conditions (28, 31). It is not clear how aging affects the nitric oxide-cGMP signal transduction pathway.

We tested the hypothesis that the nitric oxide-induced negative functional effect on cardiac myocytes would be reduced by aging. We examined whether this was related to a decreased action of the cGMP-dependent protein kinase in aged ventricular myocytes. We studied the effects of S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide donor, and KT-5823, a cGMP protein kinase inhibitor. We determined the nitric oxide-induced contractile response of ventricular myocytes from young and old rabbits and the effect of the cGMP protein kinase on the response to nitric oxide in ventricular myocytes from young and old rabbits.

AGING CAUSES CHANGES in structure and function of the heart and blood vessels, which leads to an increase in the incidence of certain cardiovascular diseases such as coronary artery disease, heart failure, hypertension, and myocardial infarction (2, 16, 19). The effects of aging on cardiac myocytes include a reduced ability to use energy and perform work as well as alterations in ion currents (6, 7, 14, 16). Furthermore, a decrease in the myocardial sarcoplasmic reticulum calcium content, a prolongation of contraction, and a decrease in sensitivity to β-adrenergic stimulation have also been observed with age (5, 13, 16).

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**METHODS**

**Cell dissociation.** The investigation was conducted in accordance with the National Institutes of Health Guide for the Care of Laboratory Animals (DHHS Publication 85-23, Revised 1996) and was approved by our Institutional Animal Care and Use Committee. Ventricular myocytes were isolated from hearts of New Zealand White rabbits as previously described (10, 11). Briefly, the rabbits were anesthetized with pentobarbital sodium (35 mg/kg) followed by the administration of heparin (10 U/g body wt) intravenously with the use of circumflex ear vein. The heart was immediately removed after an overdose of pentobarbital (60 mg/kg) and retrograde perfused through the aorta with minimal essential medium (MEM, Sigma) supplemented with 10 mM taurine, 2 mM L-glutamic acid, and 20 mM HEPES, pH 7.2. After 5 min of perfusion with MEM, the heart was perfused with MEM containing 0.1% type II collagenase (Worthington) for 16 min. All perfusion media were maintained at 37°C and equilibrated with water-saturated oxygen. After collagenase perfusion, the heart was removed from the perfusion apparatus, and the ventricle was cut into 8–10 pieces. The tissue suspension was further treated with MEM containing 0.1% collagenase, 0.5% bovine serum albumin (BSA), fraction V (Sigma) at 37°C and gently swirled at 2 cycles/s for 5 min. A slurry containing isolated myocytes was decanted from the tissue suspension. The isolated cells were washed three times in MEM containing 0.5% BSA and centrifuged at low speed (34 g) to remove the collagenase and subcellular debris. Incubation of the remaining tissue with collagenase was repeated at least two more times. Myocyte viability was assessed by maintenance of a rod-shaped morphology and was between 50 and 70%. Yields were typically 10–14 × 10⁶ rod-shaped cells/heart.

**Myocyte functional measurements.** Individual ventricular myocytes were studied for function. Cells were suspended in 2 ml of 2 mM Ca²⁺ MEM solution maintained at 37°C in a chamber that was fitted onto the stage of an inverted microscope (Zeiss Axiovert 125, Carl Zeiss). Two platinum wires were inserted into two parallel sides of the chamber and were used to pace the myocytes by electric field stimulation (1 Hz, 5 ms duration, voltage 10% above threshold, and polarity altered with each pulse). Unloaded shortening of selected cardiac myocytes was measured online by using a video edge-detector system (model VED-114, Crystal Biotech, Potton Biomedical) and a camera (Pulnix, TM-640), which detected the change of position of both edges of the cell. Data were collected continuously. The output of the video edge detector was fed into a television monitor and a desktop computer, which was used to analyze the data. Cells used to determine the functional parameters were rod shaped and could react to different reagents throughout the experiment. Cell contraction measurements were obtained on random cells in each preparation, and each cell was required to complete its protocol. Untreated cells continued to contract at a constant level during the experiment.

**Experimental protocol.** Myocytes from old (3 yr, n = 7) and young (6 mo, n = 6) rabbits were used in the following protocol for cell functional measurements. In all groups, myocytes were suspended in a chamber with 2 ml of MEM containing 2 mM Ca²⁺ and 0.5% BSA. After a 5-min stabilization period paced with electrical field stimulation, baseline contraction data for individual myocytes were recorded. At 5-min intervals, reagents were added to the medium, and cell contractility was measured. In both the young and aged groups SNAP was added at series concentration 10⁻², 10⁻⁶, and 10⁻⁵ M followed by KT-5823 10⁻⁶ M, a cGMP-protein kinase inhibitor. A minimum of 10 consecutive contractions was used for each data point. For each protocol at least three cells in each animal were repeatedly measured. Measurements obtained included resting cell length, absolute cell shortening, maximal rate of shortening, rate of cell relaxation, and calculated percentage of cell shortening.

**Myocyte extract preparation.** Ventricular myocytes were obtained from 3-yr-old (old) and 6-mo-old (young) healthy New Zealand White rabbits. Cells from four young and four old rabbits were studied. A suspension of myocytes was centrifuged. The pellet was collected and frozen immediately in liquid nitrogen. The frozen cells were stored at −80°C. Myocytes were homogenized for 15 s at 20,000 rpm (Polytron homogenizer) in homogenizing buffer (0.25 M sucrose, 5 mM Tris, pH 7.4, 1 mM MgCl₂) and centrifuged at 15,500 rpm at 4°C for 20 min. The supernatant was collected and used as a myocyte extract for phosphorylation reactions. Protein concentration of each sample was determined by the Bradford dye-binding procedure using a spectrometer at 595 nm. BSA was used as standard. Cell extract aliquots were stored at 80°C.

**In vitro phosphorylation reaction.** All reactions were carried out in microfuge tubes at room temperature. The protein concentrations of the myocyte extract from young and old rabbits were equalized to 0.9 mg/ml. The reaction mixture included 10 μl of protein extract, 0.4 mM 8-bromo-cGMP with or without 0.4 mM KT-5823, and 0.5 μl [γ⁻³²P]ATP (10 μCi/μl, Amersham) with total volume of 40 μl. The reaction lasted for 15 min and was terminated by adding 20 μl of Bio-Rad reducing sample buffer. The samples were heated at 95°C for 5 min, and an equal amount of protein was loaded onto each lane. Proteins were separated by electrophoresis using a miniature 12% SDS-polyacrylamide slab gel system. The gels were then stained with 0.1% Coomassie brilliant blue R-250 and destained. Gels containing phosphoproteins were dried on chromatography paper, and autoradiography was performed.

**Phosphoprotein analysis and densitometry.** The phosphoprotein bands were evaluated by densitometry to determine the amount of [³²P]phosphate incorporated into the phosphorylated proteins. The amount of phosphoproteins was determined by Bio-Rad Imaging densitometer (model GS-670) and analyzed with Molecular Analyst Software by Bio-Rad. For each band of a specific molecular weight, densitometry was used to evaluate the amount of [³²P]phosphate incorporated. Four samples from different animals were repeatedly measured to obtain the average the degree of phosphorylation.

**Statistics.** Results are expressed as means ± SE. A repeated-measures analysis of variance (ANOVA) was used to compare variables measured during the experimental and control conditions. Duncan’s multiple range test was used to compare difference between baseline and various treatments. In all cases, a value of P < 0.05 was accepted as significant. The Wilcoxon Rank-Sum test was used to analyze the densitometry data.

**RESULTS**

In the 6-mo-old rabbits, the heart weight-to-body weight ratio was 2.07 ± 0.9 g/kg (n = 6). In the 3-yr-old rabbits, the heart weight-to-body weight ratio was 2.15 ± 0.5 g/kg (n = 7). There was no statistical difference in the ratio of heart weight to body weight between the two groups. The length of the ventricular myocytes from young and old animal was also similar.
treated with SNAP (10^{-7}, 10^{-6}, and 10^{-5} M) was added to cells, the percent shortening decreased in a concentration-dependent manner by 17%, 23%, and 28% (Fig. 1). The percent shortening was significantly lower in the presence of 10^{-6} and 10^{-5} M of SNAP than that of baseline. The subsequent addition of KT-5823 (10^{-5} M) significantly increased the percent shortening from this level (SNAP 10^{-5} M). In the young myocyte group, the percent shortening started at a similar base level to that of the young myocyte group (young: 5.3 ± 0.4%, old: 5.2 ± 0.7%). The percent shortening also decreased when treated with SNAP (10^{-7}, 10^{-6}, and 10^{-5} M) by 14%, 17%, and 23% (Fig. 1). The percent shortening with SNAP 10^{-5} M was significantly lower than baseline and similar to the comparable young data. The addition of KT-5823 did not reverse the reduction of percent shortening by SNAP compared with SNAP (10^{-5} M) in the old group.

Baseline measurements of maximum shortening velocity of myocytes from young and old animals started at a similar level (young: 86.2 ± 5.3 μm/s, old: 86.4 ± 8.3 μm/s). In the young myocyte group, maximal rate of shortening ($R_{\text{max}}$) was decreased significantly by 25% when 10^{-5} M SNAP was present (Fig. 2). Addition of the cGMP protein kinase inhibitor KT-5823 (10^{-6} M) increased $R_{\text{max}}$ toward baseline. This value was significantly higher than that of cells treated with SNAP (10^{-5} M). In the corresponding old myocyte group, SNAP also reduced maximal rate of myocyte shortening by 24% when SNAP (10^{-5} M) was added. However, the addition of KT-5823 did not significantly change $R_{\text{max}}$.

The effects of SNAP and KT-5823 on the maximal rate of relaxation of young and old myocytes were similar to those for percent shortening and maximal rate of shortening. In young myocytes, the maximal rate of relaxation was decreased in a concentration-dependent manner and decreased by 23% when 10^{-5} M SNAP was added (Table 1). Addition of 10^{-6} M KT-5823 increased the maximal rate of relaxation significantly above the 10^{-5} M SNAP-treated myocytes. In contrast, whereas maximal rate of relaxation in old myocytes also decreased in a concentration-dependent manner with SNAP, decreasing by 24% when 10^{-5} M SNAP was present, KT-5823 did not affect the SNAP-induced reduction (Table 1).

SNAP significantly increased time to peak shortening in the young myocytes at a concentration of 10^{-5} M (Table 2). No other dose of SNAP or the addition of KT-5823 significantly affected the time to peak shortening in the young myocytes. In the old myocytes, neither SNAP nor KT-5823 significantly altered the

![Fig. 1. Effects of SNAP and KT-5823 on percent shortening of ventricular myocytes from young and old rabbit hearts. Basal percent shortening was similar in myocytes from young and old rabbits. Response to SNAP was also similar in young and old myocytes. KT-5823 partially reversed the effect of SNAP in young myocytes. However, KT-5823 did not significantly affect the old myocytes. *Significantly different from control level. †Significantly different from myocytes treated with 10^{-5} M SNAP.](Image 60x132 to 301x311)

![Fig. 2. Effects of S-nitroso-N-acetylpenicillamine (SNAP) and KT-5823 on the maximum rate of shortening of ventricular myocytes from young and old rabbit hearts. Basal maximum rate of shortening was similar in myocytes from young and old rabbits. The response to SNAP was also similar in young and old myocytes. KT-5823 partially reversed the effect of SNAP in young myocytes. However, KT-5823 did not significantly affect the old myocytes. *Significantly different from control level. †Significantly different from myocytes treated with 10^{-5} M SNAP.](Image 318x543 to 571x725)

Table 1. Effects of SNAP and KT-5823 on the maximal rate of relaxation of ventricular myocytes isolated from young and old rabbits

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>82.6±5.2</td>
<td>85.2±7.5</td>
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<tr>
<td>SNAP</td>
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<td></td>
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<tr>
<td>10^{-7} M</td>
<td>71.1±5.3</td>
<td>70.9±6.3</td>
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<tr>
<td>10^{-6} M</td>
<td>67.0±5.3*</td>
<td>72.4±9.0</td>
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<tr>
<td>10^{-5} M</td>
<td>63.9±4.4*</td>
<td>64.9±7.3*</td>
</tr>
<tr>
<td>KT-5823, 10^{-6} M</td>
<td>69.1±5.2</td>
<td>64.1±6.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in μm/s). SNAP, S-nitroso-N-acetylpenicillamine. *Significantly different from control. †Significantly different from SNAP (10^{-5} M).
Table 2. Effects of SNAP and KT-5823 on the time to peak shortening of ventricular myocytes isolated from young and old rabbits (Values are means ± SE (in s). *Significantly different from baseline.)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>0.37 ± 0.02</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>SNAP</td>
<td></td>
<td></td>
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<tr>
<td>10⁻⁷ M</td>
<td>0.40 ± 0.02</td>
<td>0.42 ± 0.03</td>
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<tr>
<td>10⁻⁶ M</td>
<td>0.43 ± 0.02</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>KT-5823, 10⁻⁶ M</td>
<td>0.43 ± 0.01*</td>
<td>0.42 ± 0.03</td>
</tr>
</tbody>
</table>

Table 3. Effects of SNAP and KT-5823 on the time to 90% relaxation of ventricular myocytes isolated from young and old rabbits (Values are means ± SE (in s). *Significantly different from SNAP (10⁻⁵ M). †Significantly different from old myocytes.)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.30 ± 0.02</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>SNAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>0.34 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>0.31 ± 0.01</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>0.34 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>KT-5823, 10⁻⁶ M</td>
<td>0.30 ± 0.01*†</td>
<td>0.35 ± 0.01</td>
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DISCUSSION

In this study we observed that SNAP, a nitric oxide donor, reduced the shortening of ventricular myocytes from both young and old animals. KT-5823, a selective inhibitor of cGMP-dependent protein kinase, partially reversed the SNAP-induced reduction in myocyte function in young myocytes but not in old myocytes. The effects of cGMP on protein phosphorylation were also significantly reduced in old myocytes. This suggested that the cGMP-mediated nitric oxide signaling pathway was altered in old myocytes.

One of the major actions of nitric oxide is to activate soluble guanylyl cyclase and increase the level of cGMP. Both nitric oxide and cGMP have been shown to elicit negative functional and metabolic effects in isolated cardiac myocytes as well as in the intact heart (4, 18, 20). One of the effects of cGMP signaling is mediated through a decrease in cytosolic Ca²⁺ by activation of Ca²⁺-ATPase and the subsequent removal of Ca²⁺ from the myocytes (6, 22). Some of the effects of cGMP act through protein phosphorylation by cGMP-dependent protein kinase and cGMP-regulated cAMP phosphodiesterases (18, 21, 31). Previous work demonstrated that the cGMP-dependent protein kinase was a major pathway to mediate the negative metabolic and functional effects of cGMP (21, 26, 28). We evaluated the importance of the cGMP protein kinase in the nitric oxide pathway and the changes associated with aging. Consistent with previous observations, our results showed that nitric oxide reduced myocyte function in young ventricular myocytes and that the nitric oxide-induced negative effects were partially mediated.
through the activation of the cGMP protein kinase (10, 20, 28).

We used ventricular myocytes from New Zealand White rabbits to study the effects of aging. This prevented interaction with other cell types. We examined 6-mo-old rabbits and 3-yr-old rabbits. These animals had similar heart weight-to-body weight ratios and myocyte size. The use of isolated ventricular myocytes assures that cells are homogenous, experimental conditions are standardized, and results are consistent. The yields were high with 50–70% of rod-shaped healthy cells and not different between groups. The viability of the cells was confirmed by observing their shape and contractility. The cells were able to contract for 30 min in the steady state (baseline level) in the absence of any reagents. With regard to the functional measurements, we only used cells that completed the entire protocol. Functional measurements were performed on three cells per group per heart. For the phosphoprotein analysis, frozen cells from each experimental animal were kept in a −80°C freezer, which should prevent the proteins from degrading.

The effects of aging on the heart have been shown to be associated with decreased sensitivity to β-adrenergic stimulation (13, 16). Recent research also suggested that there was a shift in the myosin isoform from α-myosin heavy chain to β-myosin heavy chain in aged cardiac myocytes, which may be responsible for the slowing of the rate sarcomere shortening and the rightward shift of the tension-pCa relation (8, 30). Other changes such as prolongation of contraction, decreased time to peak tension, and reduced contractile response by IGF-1 have also been reported (2, 9, 23). However, with the use of a carefully selected model Fischer 344 × Brown Norway rats for the study of aging, it was found that changes in relaxation seen in other rat strains might result from compensatory mechanisms induced by pathological conditions. The primary effect of aging in isolated cardiac myocytes was only marginally significant (30). We found that old myocytes had relatively similar basal function and responded similarly to SNAP compared with the young myocytes.

Nitric oxide-induced negative inotropic effects on cardiac myocytes act largely through activation of cGMP-dependent mechanisms. It has been suggested that nitric oxide and the cGMP pathway play a significant role in producing some of the aging-induced changes observed in the heart (19, 25, 29). Basal and stimulated release of nitric oxide by the coronary endothelium was reduced in aged animals (1, 29). However, IGF-1 induced cardiac contractile responses were reduced with advanced age, and this may be related to an increase in the importance of nitric oxide in the aged myocardium (23, 24).

Our results showed that differences between the young and old ventricular myocytes in response to SNAP were modest. SNAP reduced the percent shortening, maximal rate of shortening, and maximal rate of relaxation to a similar extent in both young and old myocytes. There was only a very marginally reduced response in older ventricular myocytes. In the young myocytes, when the cGMP-dependent protein kinase was suppressed by KT-5823, the maximum rate of shortening and relaxation and percent shortening were increased. In the old myocytes, KT-5823 had no significant effect on myocyte function. This indicated that in young myocytes, the effects of nitric oxide were at least partially mediated via cGMP and the cGMP protein kinase. This effect of the cGMP protein kinase was absent in the older myocytes. The findings in the young myocytes that cGMP increased protein phosphorylation and KT-5823 reduced this effect were consistent with previous observations that similar proteins were phosphorylated by cGMP (10). These proteins were also phosphorylated in the older myocytes but to a significantly lesser extent. Decreased cGMP-dependent protein phosphorylation in old myocytes suggests reduced kinase activity in the cGMP signaling pathway in old myocytes. These phosphoproteins have not yet been identified. They may play an important role in nitric oxide-cGMP signaling in the control of young cardiac myocytes’ function.

These results suggested that SNAP-induced decreases in young myocyte contractility were partially mediated through cGMP signaling and the action of the cGMP protein kinase. In old myocytes, KT-5823 did not reverse the negative effects of nitric oxide, suggesting that the cGMP-cGMP protein kinase pathway was impaired. However, SNAP also decreased old myocyte contraction, implying that this decreased myocyte contraction in old animals was mainly due to non-cGMP protein kinase pathways. It has been suggested that cGMP, via the cGMP-dependent protein kinase, affects cytosolic calcium (6, 14, 26). Some of the effects of nitric oxide on calcium level may be independent of the cGMP protein kinase (11, 20, 32). It has also been reported that other pathways of cGMP in cardiac myocytes are mediated through cGMP-gated ion channels and cGMP-regulated phosphodiesterases (18, 20, 31). Nitric oxide may also directly modulate protein function in the heart in a cGMP-independent manner, e.g., via trans-nitrosylation of regulatory thiols (27, 33). It is possible that one of these pathways was enhanced in the aged myocytes.

In summary, we observed that nitric oxide reduced the function of ventricular myocytes from both young and old animals to a similar extent. Inhibition of the cGMP-dependent protein kinase partially reversed the nitric oxide-induced reduction in myocyte function in young myocytes but not in old myocytes. The effects of cGMP on protein phosphorylation were also significantly reduced in old myocytes. This suggested that the cGMP-mediated nitric oxide signaling pathway was altered in old myocytes to help maintain its function. Other pathway(s) may compensate to allow maintenance of the functional response of aged myocytes to nitric oxide.

This study was supported, in part, by a grant from the National Institute on Aging Grant AG-17648.
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AJP-Heart Circ Physiol • VOL. 281 • DECEMBER 2001 • www.ajpheart.org