Nitric oxide signaling in stretch-induced apoptosis of neonatal rat cardiomyocytes

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ABSTRACT Pressure overload associated with hypertension is an important pathological factor leading to heart remodeling and ultimately heart failure partially due to cardiomyocyte apoptosis. Here we show that endogenous NO signaling plays a critical role in mechanical stretch-induced cardiomyocyte apoptosis. Mechanical stretch induced elevated expression of both eNOS and inducible NO synthase (iNOS) and increased synthesis of NO. A sustained increase in iNOS expression was also found in hearts of hypertensive rats in vivo. Blockade of NO signaling by inhibitors of NOS (L-NAME and AMT) or downstream guanylyl cyclase (ODQ) strongly inhibited stretch-induced apoptosis, suggesting that NO is required in stretch-induced cardiomyocyte apoptosis. The expression of iNOS, but not eNOS, was blocked by L-NAME and ODQ, indicating that the iNOS induction is NO dependent. The initial elevation of NO is likely due to Ca2+-dependent activation of eNOS because elimination of intracellular calcium by EGTA-AM inhibited both iNOS induction and NO elevation. Other calcium signaling inhibitors (nifedipine, ryanodine, thapsigargin, and ionic gadolinium) also attenuated the initial NO elevation. These data indicate that mechanical signals initiate Ca2+-dependent NO synthesis, which is further amplified by activation of NO-induced iNOS expression, to regulate cardiomyocyte apoptosis.—Liao, X., Liu, J-M., Du, Lei, Tang, A., Shang, Y., Wang, S. Q., Chen, L-Y, Chen, Q. Nitric oxide signaling in stretch-induced apoptosis of neonatal rat cardiomyocytes. FASEB J. 20, E1196–E1204 (2006)

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Abnormal mechanical load as seen in hypertension leads to heart remodeling and ultimately failure (1,2). Heart remodeling results from cardiomyocyte hypertrophy, cardiac fibroblast proliferation, and cardiomyocyte loss. Accumulating evidence suggests that the loss of functional cardiomyocytes due to apoptosis plays a critical role in the transition from hypertensive heart remodeling to heart failure (3, 4). Apoptosis has been recognized as a major mechanism in the development of a number of cardiovascular diseases, including hypertension (5–7). However, the mechanisms by which the mechanical signals lead to apoptosis are still obscure.

Mechanical stretch was reported to activate the rennin-angiotensin system (RAS) in neonatal rat ventricular myocytes (NRVMs) to induce apoptosis in a p53-dependent manner (8). However, there is evidence that argues against the requirement of RAS in regulating stretch-induced responses in cardiomyocytes (9). p53 may not be necessary for stretch-induced cardiomyocyte apoptosis since p53 is induced relatively late after stretch (10). The reactive oxygen species (ROS) were also reported to be critical for stretch-induced cardiac hypertrophic and apoptotic responses (11), but how ROS signaling was initiated by stretch and how it regulated apoptotic pathway are unknown. Recently, we and others reported that mechanical stretch activated Ca2+ signaling in cardiomyocytes (12, 13), and this Ca2+ signaling played an essential role in apoptosis initiation (12). However, the downstream signals that couple Ca2+ signaling to the apoptotic machinery remain to be elucidated.

In this study, we demonstrate that nitric oxide (NO) is likely to be the link between stretch-induced Ca2+ signaling and apoptosis in cardiomyocytes. We found that mechanical signal initially activated a Ca2+-dependent NO synthesis, which was further amplified by activation of NO-induced iNOS expression, to regulate cardiomyocyte apoptosis.
MATERIALS AND METHODS

Materials

Nitric oxide fluorescent probe DAF-FM DA (4-amino-5-methyl-ylamino-2′,7′-difluorofluorescein diacetate), mitochondrial membrane potential indicator DIOC6(3) (3,3′-dihexyloxacarbocyanine iodide), and EGTA-AM (EGTA Acetoxyethyl ester) were from Molecular Probes (Eugene, OR, USA). Nitrate reductase-based NO detection kit and immunohistochemistry avidin-biotin complex (ABC) kit were from Jingmei BioTech Co. Ltd (Beijing, China). DAPI (4,6-diamidino-2-phenyldole), EDTA, polyacrylamide gel reagents, bromodeoxyuridine (BrdU) (5-bromo-2′-deoxyuridine), arginine, and anti-β-actin monoclonal antibody (mAb) (Sigma-5316) were from Sigma (St. Louis, MO, USA). Trypsin, Dulbecco’s modified Eagle medium (DMEM)-F12 medium, and TRizol reagent were products of Invitrogen (Carlsbad, CA, USA). FBS was from Hyclone (Logan, UT, USA). Anti-inducible nitric oxide synthase (NOS) (BD610296), and anti-cyt c (BD556432) monoclonal antibodies were from BD Biosciences (Franklin Lakes, NJ, USA). Horseradish peroxidase (HRP)-labeled secondary antibodies were from KPL (Gaithersburg, MD, USA). SNAP (S-nitroso-N-acetylpenicillamine), AMT ((±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, HCl), L-NNAME (N-nitro-L-arginine-methyl ester), and ODQ (1H-[1,2,4]-oxidiazolo[4,3-A]-quinoxalin-1-one) were products of CalBiochem (Darmstadt, Germany). In Situ Cell Death Detection Kit was purchased from Roche Diagnostics (Indianapolis, IN, USA).

Cell preparation and in vitro stretch model

NRVMs were isolated from the hearts of Wistar rats (0–3 days) and seeded in stretch chambers as described previously (12, 14). After 24 h serum starvation, the medium was changed, and the cells were subjected to 20% sustained stretch. Cells cultured under identical conditions without stretch were used as controls.

Animal model of hypertension

All animal studies were approved by Cardiovascular Institute Committee for the Care of Experimental Animals. Hypertensive rat model was produced in Wistar rats (male, 250 to 300 g, 8 to 10 wk old, supplied by the Experimental Animals Institute of Chinese Academy of Medical Sciences, Beijing, China) with abdominal aortic constriction (AAC) as described before (15). Rats in control group were sham-operated following a similar protocol, leaving the abdominal aorta intact. Carotid blood pressure was recorded in narcotized rats to monitor hypertension. Ventricles of AAC hearts as well as sham-operated controls were removed and used for immunohistochemistry and Western blot.

Intracellular NO detection

Intracellular NO level ([NO] i) was measured by confocal microscopy as described (12), using a NO-sensitive fluorescence probe DAF-FM (16). Briefly, cells were loaded with DAF-FM DA (10 μmol/l) at 37°C for 30 min in Tyrode buffer (in mmol/l: 137 NaCl, 5.4 KCl, 20 HEPES, 1.2 MgCl2, 1.0 CaCl2, 10 glucose (Glc)), pH7.4), then gently washed twice and incubated for another 30 min to achieve complete cleavage of DAF-FM DA by the intracellular ester enzyme that releases the NO-sensitive probe (DAF-FM). Fluorescence was detected with a laser scanning confocal microscope (Zeiss LSM 510) in Tyrode buffer containing arginine (100 μmol/l) at room temperature. [NO] i was calculated using the same approach as described for [Ca2+] i (12).

Extracellular nitrate detection

Extracellular total nitrate level ([NO] t) was determined by a nitrate reductase-based colorimetric kit. NO and NO-derived nitrate was converted to NO3− by nitrate reductase, followed by the spectrophotometric quantification of nitrite levels using Griess Reagent. KNO3 (100 μmol/l) was used as nitrate standard.

Immunohistochemistry

Immunohistochemistry of iNOS and eNOS was performed following the regular protocol (17). Briefly, deparaffinized sections were blocked in 10% normal goat serum, then incubated with antibody iNOS or eNOS, 1:200 dilution at 37°C for 1 h. Slides were washed and incubated with biotinylated secondary Ab. After treatment of slides with ABC kit, antigens were visualized with 3,3-diaminobenzidine (DAB) system.

Apoptosis assays

Apoptosis in cell culture was examined by annexin V-based flow cytometry and/or in situ DAPI staining as described (12). For flow cytometry, cells were stained with annexin V-FITC (25 ng/ml) simultaneously with PI (10 ng/ml) and the apoptotic population (annexin V+/PI−) was analyzed using a FACScan (BD) with CellQuest software. For DAPI staining, adhered cells in stretch chambers were fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.1% Tween-20, then stained with DAPI (10 ng/ml). DAPI fluorescence was visualized with fluorescent microscope under UV excitation. Apoptotic cells showed nuclear condensation and fragmentation.

Apoptosis in rat heart in vivo was determined by in situ TUNEL assay following the manufacturer’s standard protocol. Briefly, 4% paraformaldehyde fixed cardiac tissues were paraffin embedded. After dewaxation, rehydration, and proteinase K treatment, samples were incubated with TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. The slices were rinsed with PBS, then incubated with 50 μl converter POD in a humidified chamber for 30 min at 37°C. After DAB substrate treatment, the slices were mounted under glass coverslip and analyzed under a light microscope.

Western blot

Western blot was performed as described earlier (10). Cells were lysed in lysis buffer (in mmol/l: 25 HEPES, pH7.4, 5 EDTA, 8.0 EGTA, 1.0 Na3VO4, 0.25 NaF, 0.1 phenylmethylsulfonyl fluoride, 1.0 dithiothreitol, and 1% Nonidet P-40, 5 μg/ml aprotinin, 100 μg/ml leupeptin, 50 μg/ml trypsin inhibitor). Cellular protein (50 μg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane by standard electric transfer protocol. The membrane was probed with primary antibodies, then with HRP-labeled secondary Ab. Immunoreactive bands were visualized with ECL reagent.

Cell fractionation

Cell fractionation was performed as described before (10). Briefly, 1 × 106 cells were gently homogenized (5–10 strokes)
with a Dounce homogenizer in buffer A (in mmol/l: 20 HEPES-KOH, pH7.2, 10 KCl, 1.5 MgCl₂, 1.0 EDTA, 1.0 EGTA, 250 sucrose, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 100 µg/ml leupeptin, 50 µg/ml trypsin inhibitor) The homogenate was centrifuged at 750 g for 5 min at 4°C and the supernatant was subjected to further centrifugation at 10,000 g for 10 min at 4°C. The supernatant from this step was subjected to ultracentrifugation at 100,000 g for 45 min at 4°C, and the resulting pellet and supernatant were designated P100 and S100, respectively.

Mitochondria membrane potential (ΔΨm) assay

The mitochondria membrane potential (ΔΨm) was measured by DiOC₆(3)-based flow cytometry as described (10). DiOC₆(3) (20 nmol/l) was loaded into 4 × 10⁵ cells suspended in 0.5 µl fresh DMEM (pH7.2) and fluorescence was examined at 530 ± 30 nm (FL1 of BD FACScan). Data were obtained and analyzed using CellQuest software from a PI negative cell population.

Reverse transcription-polymerase chain reaction (RT-polymerase chain reaction)

Total RNA was isolated from cultured NRVMs using the TRIzol reagent and 5 µg RNA was reverse transcribed (RT) using oligo-dT. Two microliters of cDNA were amplified by polymerase chain reaction (PCR) using the following primers: iNOS, 5'-CCT AAG AGT CAC AAG CAT C-3'/5'-CTA TTT CCT TGG TTA CCG C-3'; eNOS, 5'-CGA GAT ATC TTC AGT CCC AAG C-3'/5'-AGG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ATG GTG GTG AGT TCC ACC ACC CTG TTG CTG TA-3'.

Statistical analysis

All results were expressed as the mean ± sd of at least 3 independent experiments unless stated otherwise. Significance was determined with the Student’s t test. Difference between groups were considered significant at a value of P < 0.05.

RESULTS

Stretch-activated NO synthesis in NRVMs

Fluorescent microscopy data indicated that a 20% sustained stretch induced an increase in [NO]i within 5 min as demonstrated by an increase in DAF-FM fluorescence intensity (Fig 1A). The correlation between DAF-FM fluorescence and [NO]i was confirmed by the data from SNAP (a NO donor) -treated cells, which showed stronger fluorescence (Fig 1A). Stretch-induced [NO]i elevation was cardiomyocyte-specific since there was no detectable change of [NO]i in cardiac fibroblasts (data not shown). Quantification of [NO]i demonstrated that stretch induced rapid and significant [NO]i elevation (135±8% at 5 min, 121±7% at 10 min vs. 100% at 0 min, P < 0.05) in NRVMs (Fig 1B). After the initial transient elevation, [NO]i tended to recover but was maintained at a higher level than that of control cells (106±3% at 40 min vs. 100% at 0 min, P < 0.05) (Fig 1B).

Microscopic methods are not useful in examining a long-term stretch effect. Therefore, we used another nitrate-reductase based method to detect NO after long-term stretch. Consistent with short-term stretch, there was an ~3-fold increase in total nitrate ([NO3]t) detected in 24 h stretched cardiomyocyte compared to unstretched control (81.0±6.6 vs. 28.5±2.4, µmol/l...
\[ [\text{NO}^{-3}] \leq 0.05 \] (Fig 1C). This result indicated that stretch-induced NO synthesis was sustained, although the initial [NO\textsubscript{i}] elevation was transient.

L-NAME, a nonisoform-specific NOS inhibitor, completely abrogated stretch-induced [NO\textsubscript{i}] elevation, which suggested that stretch-induced elevation of [NO\textsubscript{i}] was due to de novo NO synthesis (Fig. 1D). However, AMT, an iNOS-specific inhibitor, failed to block the initial phase of stretch-induced NO elevation (Fig 1D), indicating that iNOS was not responsible for this portion of NO production. It is well known that Ca\textsuperscript{2+}-dependent eNOS is constitutively expressed in cardiomyocyte (18) (also see Fig 1E). On the other hand, iNOS is Ca\textsuperscript{2+} independent and appears to be regulated at the transcriptional level (19). Therefore, we used EGTA-AM to eliminate the intracellular Ca\textsuperscript{2+} signal to distinguish roles of eNOS and iNOS in stretch-induced initial phase of NO synthesis. EGTA-AM treatment significantly inhibited stretch-induced [NO\textsubscript{i}] elevation (at 10 min), indicating the initial NO synthesis was Ca\textsuperscript{2+} dependent (Fig 1D). Together with the basal expression levels of eNOS and iNOS in cardiomyocyte (Fig 1E), these data demonstrated that stretch-induced initial [NO\textsubscript{i}] elevation was due to Ca\textsuperscript{2+}-dependent eNOS activation. Mechanical stretch also induced eNOS and iNOS expression in NRVMs as revealed by Western blot (Fig 1E).

**NOS expression and apoptosis in hearts of hypertension rats**

Since we found stretch induced significant induction of iNOS \textit{in vitro}, we checked abdominal aorta constricted (AAC) hypertensive rats to confirm whether \textit{in vivo} mechanical overload affected NOS expression in heart. We found that both eNOS and iNOS levels began to increase in hearts of AAC rats from 24 h after surgery compared to sham-operated controls, as revealed by immunohistochemistry (Fig 2A). Although eNOS expression returned to basal level within 7 days, iNOS induction remained significantly high after 2 wk from surgery (Fig 2A). Western blot analysis also showed that pressure overload induced expression of iNOS, but not eNOS, after 7 days of AAC operation (Fig 2B). The hypertension model was confirmed by monitoring rat mean arterial pressure (MAP). After surgery, MAP of AAC rats increased significantly within 24 h (blood pressure in mmHg: 121.0±7.5 vs. 104.5±6.3, P<0.05) and remained significantly higher (126.9±3.4 vs. 101.1±8.2, P<0.05) at 14 days after operation, despite a transient decrease at day 3 (98.8±8.7 vs. 109.2±1.7, P<0.05) (Fig 2C). In parallel with hypertension and NOS induction, there was increasing apoptosis in these AAC hypertensive hearts as revealed by TUNEL \textit{in situ} apoptosis assay (Fig 2D). These observations demonstrated that \textit{in vivo} mechanical overload indeed induced NOS expression in heart, which may have biological and pathological significance in regulating hypertension responses, including, but not exclusive to, apoptosis.

**Requirement of NO signaling in stretch-induced cardiomyocyte apoptosis**

To directly examine the role of NO in mechanical stretch-induced apoptosis, we pretreated NRVMs with L-NAME 30 min before stretch to block NO synthesis and found that L-NAME blocked stretch-induced apoptosis (Fig 3A, B). After 4 h stretch, a significant portion...
of stretched cells were annexin V+/PI− as seen in flow cytometry analysis; a similar amount of cells had condensed and fragmented nuclei, morphological hallmarks of apoptosis as shown in DAPI staining. Arrows indicated apoptotic nucleoli. B, C) Stretch-induced apoptosis was blocked by NO signaling inhibitors but enhanced by NO donor. Cells were incubated at 37°C for 30 min with L-NAME (100 μmol/l), AMT (100 μmol/l), ODQ (10 μmol/l), or SNAP (100 μmol/l), respectively, in each group before 4 h stretch. Control cells were incubated with inhibitors without stretch. Apoptotic index was shown as % of fragmented nucleoli (indicated by arrows in panel A). More than 200 nuclei from randomly selected fields (>10 fields per dish) were counted to calculate apoptosis index. *P < 0.05.

Figure 4. The roles of NO in mitochondrial apoptotic pathway and in regulating stretch-induced cardiomyocyte apoptosis. A) NO signaling inhibitors (L-NAME, AMT, or ODQ) blocked stretch-induced mitochondria depolarization as shown by DiOC6(3)-based ∆Ψm flow cytometry. Cells were incubated with inhibitors and stretched for 4 h as described in Fig. 3. Left shifting peak indicated a decrease of ∆Ψm. Data were representative of 3 independent assays. B) L-NAME, AMT or ODQ also blocked stretch-induced cyt c release from mitochondria. Cells were treated as described in panel A. Mitochondria free lysate fraction (S100) was examined by Western blot with anti-cyt c Ab. β-Actin was blotted to indicate equal loading.
showed that NO signaling mediated stretch-induced mitochondria-dependent cardiomyocyte apoptosis.

Mechanisms of NOS activation

We observed that stretch-induced NO signaling was required in apoptosis regulation in NRVMs and that the apoptotic NO was synthesized by NOS, perhaps iNOS. Therefore, we investigated the mechanisms by which iNOS and eNOS expressions were regulated. A time course study showed that iNOS induction was robust (increased with 30 min), and there was a 10-fold increase in iNOS protein expression 4 h after stretch compared to the basal level in unstretched (0 h) cells (Fig. 5A). We performed semiquantitative RT-polymerase chain reaction (RT-PCR) and found that iNOS mRNA also began to increase within 30 min after stretch (Fig. 5B). These results indicated that mechana-

The role of Ca²⁺ signaling in NO cascade

Since the initial [NO]₀ elevation was Ca²⁺ dependent (Fig. 1D), we further addressed how Ca²⁺ was involved in NO signaling. We previously demonstrated that mechanical stretch induces [Ca²⁺]ᵢ elevation, which depends on Ca²⁺ influx through L-type calcium channel (LCC), stretch-activated ion channel (SAC), and Ca²⁺-induced Ca²⁺ release (CICR) (12). We thus examined the effects of Ca²⁺ inhibitors (LCC blocker nifedipine, SAC blocker Gd³⁺, CICR inhibitors ryanodine, and thapsigargin) on NO production. These inhibitors have been shown to block stretch-induced [Ca²⁺]ᵢ elevation in cardiomyocytes (12), and here they also attenuated the initial [NO]₀ elevation (Fig 6A). EGTA-AM also attenuated stretch-induced NO production and iNOS expression (Fig. 1D, Fig. 6B). These data suggested that the stretch-induced Ca²⁺ signal was required for the initial NO burst and thus possibly acted as a trigger of the NO signaling cascade (by activating eNOS).

DISCUSSION

In this study we addressed how mechanical stretch signals apoptosis in cardiomyocytes. Our data suggested a mechanical stretch-induced Ca²⁺-NO signaling cascade in NRVMs as summarized in Fig. 7. Stretch may initiate the cascade through [Ca²⁺]ᵢ elevation and Ca²⁺-dependent eNOS activation. eNOS produces the initial NO, which is further amplified through NO-induced iNOS expression. iNOS produces a high dose of NO, which is able to regulate stretch-induced cardiomyocyte apoptosis and/or other stretch-induced responses. We presented the following lines of evidence: 1) mechanical stretch activated NOS expression and Ca²⁺-dependent NO synthesis; 2) blockage of NO synthesis or downstream cGMP signaling inhibited stretch-induced cardiomyocyte apoptosis and critical apoptotic mitochondrial events (ΔΨm depolarization, cyt c release); 3) blockage of initial NO synthesis or down-
stream cGMP signaling blocked stretch-induced iNOS expression, and deactivating iNOS inhibited stretch-induced apoptosis; and 4) mechanical overload increased transient eNOS elevation and sustained iNOS up-regulation in cardiac ventricle of hypertension rats in vivo.

NO has been reported to be a bidirectional regulator for apoptosis, as it can either promote or inhibit apoptosis in cardiomyocyte and other types of cells (20–24). NO can directly induce the nitrosylation of some apoptosis regulatory molecules, such as caspase 3 and cyt c, to regulate apoptosis (25), or directly target mitochondria to induce apoptosis by inhibiting mitochondrial respiration (26). Besides the mitochondria-dependent apoptotic pathway, NO also plays important roles in death receptor-dependent apoptotic pathway (27). We have reported that both mitochondria and death receptor-mediated apoptosis were activated by mechanical stretch in NRVMs (10, 28). It is possible that NO participates in both types of apoptotic pathways (mitochondria and death receptor dependent) to regulate stretch-induced cardiomyocyte apoptosis (Fig. 7). NO can also activate soluble guanylate cyclase (sGC) to produce cGMP, and subsequently activates cGMP-dependent PKG to induce apoptosis (29). This is consistent with our observation that sGC inhibitor ODQ potently inhibited apoptotic events. However, there is a report arguing that activation of sGC/cGMP pathway by low concentrations of NO rescues cells from apoptosis (30). The controversy about the pro- or antiapoptotic effects of NO may be due to different cellular models. Although a low concentration of NO has been found to protect macrophages from apoptosis (30), NO concentration in our settings may already be high enough to promote apoptosis in cardiomyocytes. We showed both in vitro and in vivo evidence that mechanical overload significantly increased iNOS expression in cardiac cells. Overall, once activated by

![Figure 6](image-url)

**Figure 6.** Stretch-induced calcium signaling regulates NO synthesis and iNOS induction. A) Ca^{2+} signaling inhibitors attenuated the initial phase of stretch-induced NO elevation. Cells were incubated at 37°C with or without nifedipine (2 μmol/l), ryanodine (10 μmol/l), thapsigargin (10 μmol/l) or GdCl3 (100 μmol/l) for 30 min before stretch, respectively in each group. [NO]i was determined by DAF-based confocal microscopy and calculated as described in Fig. 1. Changes of [NO]i (Δ[NO]i) after 10 min stretch were compared. *P < 0.05. B) Eliminating [Ca^{2+}]i by EGTA-AM inhibited stretch-induced iNOS expression. Cells were incubated with EGTA-AM (2 μmol/l) at 37°C for 30 min before stretch to eliminate intracellular Ca^{2+}. Cells were stretched for 4 h or left free as indicated. iNOS expression was examined by Western blot. β-Actin was blotted to indicate equal loading.

![Figure 7](image-url)

**Figure 7.** Summary of hypothetic NO signaling cascade activated by mechanical stretch in cardiomyocytes. Mechanical stretch initiates the NO cascade by activating constitutive expressed eNOS through [Ca^{2+}]i elevation. The active eNOS produces a small amount NO, which further induces iNOS expression. The iNOS, once expressed, produces larger amounts of NO and thus amplifies NO signaling. NO may affect mitochondria or other apoptotic pathways to induce apoptosis. Besides this NO cascade, stretch-induced Ca^{2+} may participate in other signaling pathways. Solid lines: signaling discovered in this study. Dashed lines: possible signaling.
mechanical overload, NO signaling may have multiple targets to induce apoptosis in cardiomyocytes.

Endogenous NO biosynthesis is tightly regulated by three distinct isoforms of NOS: the neuronal (nNOS, NOS I), endothelial (eNOS, NOS III), and inducible (iNOS, NOS II) forms. It has been reported that iNOS is proapoptotic, and increased iNOS expression has been found in a number of heart diseases (22–24, 31).

Overexpression of iNOS in mouse heart results in heart block and sudden death (32), and lack of iNOS seems to be of benefit to the preservation of heart function in hypertensive mice (33). In this study we found that mechanical stretch significantly induced the expression of both eNOS and iNOS in vitro and in vivo. Notably, the in vivo increase of iNOS expression was sustained in hypertensive hearts. These results strongly suggest that iNOS plays important roles in regulating pathological outcomes of hypertension. Our data also demonstrated that iNOS-produced NO was proapoptotic, since AMT blocked apoptosis without altering iNOS expression or eNOS-produced initial NO. Although eNOS induction was transient in vitro, its activation was essential in initiating stretch-induced NO cascade, the inhibition of which resulted in blockade of iNOS gene expression. Our results are inconsistent with a previous report suggesting that stretch suppresses cytokine-induced iNOS expression in cardiomyocytes (34). The discrepancy may be due to the difference between the experimental models. The cyclic stretch in their study promoted hypertrophy rather than apoptosis (11), and we didn’t treat cells with any cytokines.

Both L-NAME and ODQ strongly suppress stretch-induced iNOS expression, indicating that iNOS expression may be regulated by a NO-dependent mechanism, as reported (25). Moreover, many signaling pathways may participate in iNOS gene regulation (35, 36), therefore, it would be interesting to examine whether mechanical stretch-induced NO can activate some transcriptional factors, such as STATs, NfκB, etc., to induce iNOS expression. The induction of eNOS is apparently regulated by a distinct pathway, since the NOs and Ca"2+ inhibitors had little effect on its expression. Induction of eNOS (activity and/or expression) has been reported in genetically engineered hypertensive rats, and is related to heart failure, but the mechanisms remain unclear (37).

The link between stretch-induced Ca"2+ signaling and NO signaling has been demonstrated clearly in the present study, as we showed that Ca"2+ inhibitors (EGTA-AM, nifidipine, Gd"3+, ryanodine, and thapsigargin) significantly attenuated stretch-induced [NO]i elevation and iNOS expression (Fig 1D, Fig. 6). Therefore, the stretch-induced Ca"2+ signal is very likely to be the trigger of the subsequent NO signaling in cardiomyocytes, and the NO signaling further amplifies itself by iNOS expression to regulate stretch-induced responses including, but maybe not exclusive to, apoptosis (Fig. 7). We previously discussed the possibility for Ca"2+ signaling to be the initiator of stretch-induced apoptosis in cardiomyocytes (12), which may regulate apoptosis through many known and/or unknown pathways (Fig. 7). Clearly, the NO signaling discovered in the present study is likely to be the most important one.

Deregulation of eNOS and iNOS has been closely associated with hypertension, myocardial infarction, heart failure, and cardiac transplantation (24, 38). It is possible that activation of NO signaling could be an adaptive response to protect cardiac cells from mechanical overload-induced damage. However, maladaptive responses with a chronically high dose of NO and enhanced iNOS expression (and the potential feedback reinforcement) could be associated with the progression of heart diseases. Indeed, chronically increased NO levels and enhanced NOS expression have deteriorating effects on heart cells by directly targeting mitochondria or other cellular organelles (27, 36). Understanding the role of NO signaling in heart diseases would be useful for the development of drugs that can selectively promote the beneficial effect of NO. [7]

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REFERENCES


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SPECIFIC AIMS

Cardiomyocyte apoptosis has been recognized as an important pathological factor in the transition from hypertensive heart remodeling to heart failure, but the mechanisms by which the mechanical signals lead to apoptosis are not well understood. Building on our previous finding that stretch-activated Ca2+/H11001 signaling plays an essential role in initiating apoptosis, we studied the downstream signals and found that NO was the link between stretch-induced Ca2+/H11001 signaling and apoptosis in cardiomyocytes.

PRINCIPAL FINDINGS

1. Activation of Ca2+-dependent NO signaling in cardiomyocytes by mechanical stretch

Mechanical stretch induced rapid and significant intracellular NO ([NO]i) elevation in cardiomyocytes (Fig. 1). [NO]i elevation remained significantly higher for up to 24 h after stretch and was sustained, although the initial [NO]i elevation was transient (Fig. 1). We reasoned that stretch-induced [NO]i elevation to activated NO synthases (NOS) since L-NAME, a nonisoform-specific NOS inhibitor, completely abrogated stretch-induced [NO]i elevation (Fig 1D). The rapid initial NO synthesis was Ca2+-dependent and significantly attenuated by EGTA-AM (Fig 1D), indicating a potential role for Ca2+-activated eNOS. The inducible NOS (iNOS)-specific inhibitor AMT failed to block stretch-induced initial NO synthesis, suggesting that the proapoptotic phase of NO was produced by iNOS. Blockage of soluble guanylyl cyclase (sGC) by ODQ inhibited stretch-induced apoptosis (Fig 2C), suggesting that the NO/cGMP pathway was involved in apoptosis regulation. All these inhibitors also blocked stretch-induced mitochondrial membrane potential (ΔΨm) reduction and cyt c release, critical events in mitochondria-dependent apoptosis. These data indicated that endogenous NO signaling was required for stretch-induced cardiomyocyte apoptosis and that iNOS played important proapoptotic roles.

2. Requirement of NO signaling in stretch-induced cardiomyocyte apoptosis

To directly examine the role of NO in mechanical stretch-induced apoptosis, we pretreated cardiomyocytes with L-NAME 30 min before stretch to block NO synthesis and found that L-NAME blocked stretch-induced apoptosis (Fig 2A, B). In addition to L-NAME, a specific iNOS inhibitor AMT also inhibited apoptosis (Fig 2C). However, AMT failed to block iNOS induction or the initial phase of NO production, which suggested that the proapoptotic phase of NO was produced by iNOS. Blockage of soluble guanylyl cyclase (sGC) by ODQ inhibited stretch-induced apoptosis (Fig 2C), suggesting that the NO/cGMP pathway was involved in apoptosis regulation. All these inhibitors also blocked stretch-induced mitochondrial membrane potential (ΔΨm) reduction and cyt c release, critical events in mitochondria-dependent apoptosis. These data indicated that endogenous NO signaling was required for stretch-induced cardiomyocyte apoptosis and that iNOS played important proapoptotic roles.

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3. Roles of eNOS and iNOS in NO cascade

Although stretch induced both eNOS and iNOS, the initiating NOS is most likely eNOS since it produced the initial phase of NO; clearly the later phase of apoptotic NO was produced by iNOS, as discussed above. The next question we asked was what signal links eNOS to iNOS. We found that NO itself was the key regulator of iNOS induction. When inhibiting the initial NO production by L-NAME, it completely blocked iNOS expression. NO regulated iNOS expression mostly likely through the sGC/cGMP pathway since ODQ blocked stretch-induced iNOS induction. There was a positive feedback loop in the stretch-induced NO cascade, in which stretch activated eNOS to initiate NO production and the initial NO induced iNOS expression to produce more NO. The high level of NO produced by iNOS, which is a much more powerful NOS than eNOS, was capable of inducing apoptosis and/or other stretch-induced responses in cardiomyocytes.

Figure 1. Mechanical stretch induces Ca$^{2+}$-dependent NO production in NRVMs. A) Intracellular NO concentration ([NO]$_i$) was examined by DAF-based confocal analysis. Images shown were taken from cells before stretch (Control), 5 min after stretch (Stretch), and 10 min after SNAP (100 μM/l) treatment (SNAP). NO donor SNAP treatment was used as a positive control. B) Confocal images were analyzed with IDL software to give quantitative NO levels. Digital images were taken from random fields (>50 pictures per time point were taken) and pixel density was calculated, normalized with control data (from 10 min to 0 min before stretch) and changes of [NO], (Δ[NO]) were expressed as changes of fluorescence (ΔF/F0). Error bars stand for s.e. *P < 0.05. C) Total nitrate level ([NO]$_t$) was determined by nitrate reductase-based colorimetric method. Conditioned medium was examined. [NO]$_t$ was converted to [NO$_3$] using KNO$_3$ (100 μM/l) as a standard. *P < 0.05. D) Cells were incubated with Ca$^{2+}$ chelator EGTA-AM (2 μM/l), NOS inhibitor L-NAME (100 μM/l), or iNOS inhibitor AMT (100 μM/l) for 30 min at 37°C before stretch. [NO]$_i$ was determined by DAF-based confocal analysis. Data of 10 min stretched cells were compared. *P < 0.05. E) Protein levels of iNOS and eNOS in NRVMs before and after 4 h stretch were detected by Western blot analysis. β-Actin was blotted to monitor equal loading.

Figure 2. NO signaling participates in the regulation of stretch-induced cardiomyocyte apoptosis. A) Apoptosis was examined with annexin V-based flow cytometry (upper panel) and DAPI-based fluorescent microscopy (lower panel). To block NO synthesis, cells were pretreated with L-NAME (100 μM/l) at 37°C for 30 min before stretch. Cells were subjected to 20% sustained stretch for 4 h before apoptosis detection. Arrows indicated apoptotic nucleoli. B, C) Cells were incubated at 37°C for 30 min with L-NAME (100 μM/l), AMT (100 μM/l), ODQ (10 μM/l), or SNAP (100 μM/l), respectively, in each group before a 4 h stretch. Apoptotic index was shown as % of fragmented nucleoli (indicated by arrows in panel A). More than 200 nuclei from randomly selected fields (>10 fields per dish) were counted. *P < 0.05.
4. The role of Ca\(^{2+}\) signaling in NO cascade

We demonstrated that stretch activated a NO cascade to induce cardiomyocyte apoptosis. The next question we asked was how this cascade was initiated. We showed that stretch-induced initial NO production was Ca\(^{2+}\)-dependent in this study, and have reported that mechanical stretch-induced \([\text{Ca}^{2+}]_i\) elevation plays essential roles in apoptosis initiation. Therefore, Ca\(^{2+}\) signaling is likely to be the initiator of the NO cascade. Stretch-induced \([\text{Ca}^{2+}]_i\) elevation was dependent on Ca\(^{2+}\) influx through L-type calcium channels (LCC) and stretch-activated ion channels (SAC), and Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). The Ca\(^{2+}\) inhibitors (EGTA-AM, LCC blocker nifidipine, SAC blocker Gd\(^{3+}\), CICR inhibitors ryanodine and thapsigargin), which have been shown to block stretch-induced \([\text{Ca}^{2+}]_i\) elevation in cardiomyocytes, significantly attenuated the initial \([\text{NO}]_i\) elevation in this study. These data suggest that the stretch-induced Ca\(^{2+}\) signal is required for the initial NO burst and possibly acts as a trigger for the NO signaling cascade by activating eNOS.

CONCLUSIONS AND SIGNIFICANCE

In this paper, we suggest that mechanical stretch induces a Ca\(^{2+}\)-NO signaling cascade to induce apoptosis in cardiomyocytes, as summarized in Fig. 3. Stretch may initiate the cascade through \([\text{Ca}^{2+}]_i\) elevation and Ca\(^{2+}\)-dependent eNOS activation. eNOS produces the initial NO, which is further amplified through NO-induced iNOS expression. iNOS produces high doses of NO, which are capable of regulating stretch-induced cardiomyocyte apoptosis and/or other stretch-induced responses. This stretch-Ca\(^{2+}\)-NO signaling cascade may be a common mechanism by which cardiomyocytes sense and transduce mechanical stretch signals into biological signals to regulate hypertension responses, including, but not exclusive to, apoptosis.

Mechanical stretch significantly induced the expression of both eNOS and iNOS in vitro and in vivo. The in vitro increase of iNOS expression was sustained in hypertensive hearts. These results strongly suggest that iNOS plays an important role in regulating the pathological outcomes of hypertension. Our data demonstrated that iNOS-produced NO was proapoptotic, since AMT blocked apoptosis without altering iNOS expression or eNOS-induced initial NO. Although eNOS induction was transient in vivo, its activation was essential in initiating the stretch-induced NO cascade because its inhibition resulted in blockade of iNOS gene expression.

NO has been reported to be a bidirectional regulator of apoptosis. NO can directly induce the nitrosylation of some apoptosis regulatory molecules, such as caspase 3 and cyt c, or directly inhibit mitochondrial respiration to induce apoptosis. Besides its role in the mitochondria-dependent apoptotic pathway, NO is also important in death receptor-dependent apoptotic signaling. We have reported that mitochondria and death receptor-mediated apoptosis were both activated by mechanical stretch in cardiomyocytes. It is possible that NO participates in both types of apoptotic pathways (mitochondria and death receptor-dependent) to regulate stretch-induced cardiomyocyte apoptosis (Fig. 3).

Deregulation of both eNOS and iNOS has been associated with a number of heart diseases. It is possible that the activation of NO signaling is an adaptive response to protect cardiac cells from mechanical overload-induced damage. However, maladaptive responses with chronically high doses of NO and enhanced iNOS expression (and the potential feedback reinforcement) could be associated with the progression of heart diseases. Understanding the role of NO signaling in heart disease would be useful for the development of drugs that can selectively promote the beneficial effects of NO.

Figure 3. Schematic summary of a hypothetical NO signaling cascade activated by mechanical stretch in cardiomyocytes. Mechanical stretch initiates the NO cascade by activating constitutively expressed eNOS through \([\text{Ca}^{2+}]_i\) elevation. The active eNOS produces a small amount of NO, which further induces iNOS expression. iNOS, once expressed, produces a larger amount of NO, thus amplifying NO signaling. Finally, NO concentration reaches a certain level that is high enough to induce apoptosis and/or other responses in cardiomyocytes. NO may affect mitochondria or other apoptotic pathways to induce apoptosis. Besides this NO cascade, stretch-induced Ca\(^{2+}\) may participate in other signaling pathways. Solid lines indicate signaling discovered in this study. Dashed lines indicate possible signaling.