Inducible nitric oxide synthase augments injury elicited by oxidative stress in rat cardiac myocytes

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Igarashi, Junsuke, Masashi Nishida, Shiro Hoshida, Nobushige Yamashita, Hiroaki Kosaka, Masatsugu Hori, Tsunehiko Kuzuya, and Michihiko Tada. Inducible nitric oxide synthase augments injury elicited by oxidative stress in rat cardiac myocytes. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C245–C252, 1998.—The effects of nitric oxide (NO) produced by cardiac inducible NO synthase (iNOS) on myocardial injury after oxidative stress were examined. Interleukin-1β induced cultured rat neonatal cardiac myocytes to express iNOS. After induction of iNOS, L-arginine enhanced NO production in a concentration-dependent manner. Glutathione peroxidase (GPX) activity in myocytes was attenuated by elevated iNOS activity and by an NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP). Although NO production by iNOS did not induce myocardial injury, NO augmented release of lactate dehydrogenase from myocyte cultures after addition of H_2O_2 (0.1 mM, 1 h). Inhibition of iNOS with N^{ω}-nitro-L-arginine methyl ester ameliorated the effects of NO-enhancing treatments on myocardial injury and GPX activity. SNAP augmented the myocardial injury induced by H_2O_2. Inhibition of GPX activity with antisense oligodeoxyribonucleotide for GPX mRNA increased myocardial injury by H_2O_2. Results suggest that the induction of cardiac iNOS promotes myocardial injury due to oxidative stress via inactivation of the intrinsic antioxidant enzyme, GPX.

heart; interleukin-1β; glutathione peroxidase; antisense oligodeoxyribonucleotide

NITRIC OXIDE (NO), first identified as an endothelium-derived relaxing factor, is now recognized as a key molecule in intra- and intercellular signal transduction. In addition to endothelial cells of the coronary artery, mammalian cardiac myocytes possess NO synthase activity (38). Rat cardiac myocytes constitutively express the endothelial isoform of NO synthase (eNOS) (3), suggesting a mechanism for modulation of contractility. In addition to eNOS, inducible NO synthase (iNOS) can be expressed in the heart. Cardiac iNOS activity was first recognized in rat heart tissue after lipopolysaccharide (LPS) treatment (38). Various extracellular stimuli such as interleukin-1β (IL-1β) (41), interferon-γ (5), LPS (38), or neurohumoral factors such as catecholamine and adenosine 3′,5′-cyclic monophosphate (19, 20) induce iNOS activity in cultured myocytes. Recent clinical studies reveal that cardiac iNOS is induced in certain pathological states including heart failure (40), dilated cardiomyopathy (10, 14), myocarditis (10), and cardiac allograft rejection (29). The role of iNOS induction in the pathogenesis of heart disease is unclear, but several studies using cultured cardiac myocytes suggest that cardiac iNOS might account for the contractility dysfunction observed in endotoxin shock (4, 7). Also, inhibition of cardiac iNOS by aminoguanidine, a relatively selective inhibitor of iNOS, markedly inhibits the progression of myocardial damage in autoimmune myocarditis in rats (22). Thus cardiac iNOS may play a pivotal role in the progress of myocardial damage in many heart diseases.

The mechanism by which iNOS exacerbates myocardial injury has not been determined. Recently, we determined that NO reduces the activity of glutathione peroxidase (GPX), an antioxidant enzyme that reduces H_2O_2 in the presence of reduced glutathione, both in vitro and in cultured cells (2). In the myocardium, GPX may play a predominant role in the scavenging of H_2O_2. Catalase is the other major H_2O_2-scavenging enzyme. However, the activity of catalase in the heart is over 100-fold lower than activity in liver, another organ that contends with oxidative stress (11). The balance between production of reactive oxygen species (ROS) and degradation of ROS by antioxidant substances is critical for the homeostasis of cardiac myocytes because of their dependence on aerobic metabolism. Oxidative stress, defined as overproduction of ROS beyond the capacity of cellular antioxidant systems, could be toxic to susceptible organs such as the heart. Evidence indicates that myocardial oxidative stress increases in heart failure (16, 33) and in ischemic heart disease (27, 45). Myocardial damage has been shown to be salvaged by supplementing antioxidants to diseased hearts (for review, see Ref. 35).

Cardiac myocytes express intrinsic antioxidant enzymes such as superoxide dismutase (SOD), GPX, and catalase (18), and numerous studies (8, 24, 28, 32, 42) have demonstrated that the activity of antioxidant enzymes in the myocardium can be increased by extracellular stimuli. Antioxidant enzyme activity may become the determinant of myocardial injury, possibly by reducing oxidative stress. Our objective was to examine the effect of iNOS activity on the activity of GPX in myocardial cells and the effect of altered GPX activity on the damage to myocardial cells produced by exposure to oxidative stress. IL-1β was used to induce iNOS in cultured neonatal cardiac myocytes, and H_2O_2 was used as an oxidative stress to the cells. Antisense oligodeoxyribonucleotide for GPX mRNA was used to examine the role of GPX in oxidative injury.

METHODS

Cell culture. Neonatal rat cardiac myocytes were isolated and cultured as described previously (13). Briefly, hearts were quickly removed from ether-anesthetized neonatal Wistar-

Kyoto rats (2–6 litters). After blood had been carefully washed out, the excised ventricles were cut into 1- to 2-mm cubes and shaken for 10 min at 37°C in 15 ml phosphate-buffered saline (PBS; 137 mM NaCl, 10.6 mM Na_2HPO_4, 2.1 mM KH_2PO_4, and 1.1 mM K_2HPO_4) containing collagenase (0.1% wt/vol). The dissociated cells were suspended in 30 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (25 mM) and fetal bovine serum (FBS; 10% vol/vol) and then preplated for 1 h to selectively separate unattached myocytes. Finally, the unattached myocytes were plated on culture dishes (60 or 100 mm in diameter) or 96-well culture plates at a density of $3.1 \times 10^4/cm^2$ at 37°C under a normoxic gas mixture (95% room air and 5% CO_2; P_{O_2} = 143 mmHg). Primary culture of cardiac myocytes was used for each experiment. The percentage of beating myocytes exceeded 90% in each preparation at the time the experiment was started. Penicillin G (400 U/ml) and streptomycin (200 μg/ml) were routinely added to each culture.

Experimental protocol 1: Enhancement of NO production by cardiac myocytes. Isolated cells were cultured in DMEM with 10% FBS and 5-bromo-2′-deoxyuridine (BrdU, 100 mM) (*medium A*) for 12–16 h at 37°C under a normoxic gas mixture. BrdU was used to inhibit the growth of contaminated cardiac nonmyocytes. Then the culture medium was switched to serum-free Eagle's minimum essential medium (MEM) containing glucose (25 mM), L-arginine (L-Arg; 0.1 mM), insulin (10 mg/ml), and transferrin (10 mg/ml) (*medium B*) (42) to exclude the effects of serum. Twelve to sixteen hours later, the medium was changed to MEM containing 0.1% bovine serum albumin (BSA) and human recombinant IL-1β (10 ng/ml), and incubation proceeded for 24 h. The effects of the presence of L-Arg (0, 0.01, 0.1, and 1 mM) or the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM) during the final incubation were also examined.

To determine the effects of NO on ROS-elicited myocardial cell injury, cardiac myocytes were treated with 0.1 mM H_2O_2 in Earle's balanced salt solution (EBSS; 116 mM NaCl, 5.4 mM KCl, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4 \cdot 7H_2O$, adjusted to pH 7.4) at 37°C under a normoxic gas mixture. EBSS was used to exclude the effects of amino acids, vitamins, and glucose on the cellular injury. One hour after treatment with H_2O_2, lactate dehydrogenase (LDH) activity in EBSS was assayed.

Experimental protocol 2: Addition of S-nitroso-N-acetyl-penicillamine to cardiac myocytes. The effects of an exogenous NO releasing agent on cardiac myocytes were examined using *S*-nitroso-*N*-acetyl-penicillamine (SNAP). After culture in *medium A* for 12–16 h, followed by culture in *medium B* for 36 h, SNAP (0.05–5 mM) in MEM was added to myocyte cultures for 1 h. SNAP decreases GPX activity of cultured cells at 1 h most effectively (2). To determine the effects of SNAP on ROS-elicited myocardial injury, 0.1 mM H_2O_2 in EBSS was added to myocyte cultures for 1 h, and LDH activity in EBSS was assayed thereafter.

Experimental protocol 3: Treatment with antisense oligodeoxyribonucleotide for GPX mRNA. To test for selective attenuation of GPX enzyme activity, the effects of antisense oligodeoxyribonucleotide (ODN) were examined. The target sequence included the open reading frame of cDNA for rat liver GPX (44), nucleotides 70–89. The antisense ODN sequence was (5′ to 3′) AGCCGAGCAGCAGACATACT. To test for nonspecific effects of oligodeoxyribonucleotide, the effects of the sense primer (5′ to 3′) AGTATGTCTGCTGCTCGGCT (sense ODN) and a scrambled primer (5′ to 3′) GAAGCCAACGGGTAACTCC (scrambled ODN) were determined. The designed sequences showed no homology with other known mammalian sequences deposited in the GenBank database, as screened using the Blast program (1). Each sequence of phosphorothioate oligonucleotide (S-oligo) was produced using a DNA synthesizer, purified, dried, resuspended in buffer [100 mM tris(hydroxymethyl)aminomethane (Tris), 2 mM EDTA, and 100 mM boric acid, pH 8.4], and quantified spectrophotometrically.

Cardiac myocytes were treated with antisense ODN (50, 100, or 200 nM), sense ODN (200 nM), or scrambled ODN (200 nM) in *medium A* for the first 12–16 h and in *medium B* for the next 36 h, at 37°C under a normoxic gas mixture. To determine the effects of ODNs on ROS-elicited myocardial injury, 0.1 mM H_2O_2 in EBSS was added to myocyte cultures for 1 h, and LDH activity in EBSS was assayed thereafter.

Northern blot analysis of cardiac iNOS mRNA expression. After 24 h of IL-1β stimulation, total RNA was isolated from cultured myocytes by the guanidinium thiocyanate extraction method (9). Total RNA (10 μg) was size fractionated by gel electrophoresis, blotted onto a nylon membrane, and then hybridized for 24 h with a cDNA probe (217 bp) labeled by the random primer method (5). The cDNA probe was generated by means of reverse transcriptional polymerase chain reaction of mRNA from rat cardiac myocytes based on the iNOS mRNA sequence of rat vascular smooth muscle cells (5). The nylon membranes were washed at 55°C with a sodium citrate-salt solution (151 mM NaCl and 17 mM $C_6H_5Na_3O_7$) containing sodium dodecyl sulfate (SDS; 0.1% wt/vol), and then exposed to Kodak X-Omat RR film for 24 h.

Western blot analysis of cardiac iNOS protein. After 24 h of IL-1β stimulation, cells were scraped off with a cell scraper into 1 ml of ice-cooled protein extraction buffer (10 mM EDTA, 0.4 mM 4-amidinophenylmethanesulfonyl fluoride, and 0.2 mM leupeptin in 50 mM Tris·HCl buffer, pH 7.5). After sonication for 15 min on ice, the protein content of the extract was determined by the Lowry method (30) with bovine serum albumin as a standard. Protein samples (10 μg) were size fractionated by gel electrophoresis, blotted onto a nitrocellulose membrane, and incubated with a monoclonal antibody against mouse macrophage iNOS. The membrane was incubated with a secondary antibody labeled with horseradish peroxidase. The iNOS signal was detected with an enhanced chemiluminescence system after exposure to Kodak X-Omat RR film for 1 min.

Measurement of NO production. NO production by endogenous cardiac myocyte iNOS was determined by the measurement of the concentration of nitrite ions in the culture medium using Griess reagent (25). The culture medium was centrifuged at 2,000 *g* for 15 min, and the nitrite concentration in the supernatant was determined with an automated analyzer. NO production by myocytes was estimated by the accumulation of nitrite during 24 h of culture and normalized to the total protein content.

Measurement of GPX activity. Twenty-four hours after addition of IL-1β, 1 h after addition of SNAP, or 2 days after addition of antisense ODN, cardiac myocytes were scraped into 1 ml of ice-cooled PBS, sonicated on ice, and then centrifuged at 2,000 *g* for 15 min. The resulting supernatants were immediately studied without freezing and thawing. The activity of GPX was assayed by the methods of Flohe and Gunzler (12). Briefly, 100 μl of enzyme sample solution were added to a reaction mixture comprising 500 μl of 0.1 M potassium phosphate buffer (pH 7.4, containing 0.1 mM EDTA), 100 μl each of glutathione reductase (2.4 U/ml derived from yeast), reduced glutathione (10 mM), NADPH (1.5 mM), and H_2O_2 (1.5 mM). Consumption of NADPH at 30°C was continuously monitored at 340 nm for 10 min by using a spectrophotometer. To obtain enzyme-dependent con-

sumption of NADPH, both H_2O_2-independent and sample-independent consumption of NADPH were subtracted. One unit of GPX activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol NADPH/min. GPX activity in the cells was expressed as milliunits per milligram protein.

Measurement of the guanosine 3′,5′-cyclic monophosphate content. Cardiac myocytes were washed with ice-cooled PBS and then incubated in PBS containing 3-isobutyl-1-methylxanthine (1 mM), a cyclic nucleotide phosphodiesterase inhibitor, for 15 min at room temperature. The medium was rapidly aspirated off, and then 1 ml HCl (0.1 mM) containing 5 mM EDTA was added. The cells were scraped off with a cell scraper, and the cell mixtures were boiled at 100°C for 3 min. After centrifugation at 20,000 *g* for 10 min, the supernatants were analyzed for guanosine 3′,5′-cyclic monophosphate (cGMP) by means of an enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit. The cell remnants were solubilized in 0.1 M NaOH containing SDS (1% wt/vol) and used for determination of the protein content.

Assessment of myocyte injury. To determine the percentage of cells injured by the treatment with H_2O_2, LDH activity in the culture medium was determined using a commercially available LDH assay kit. Briefly, a 50-μl assay mixture comprising nitrozolium blue, NAD, diaphorase, DL-lithium lactate, and Tris buffer was added to 50 μl of the supernatant from a myocyte culture. After the assay mixture was incubated at 37°C for 30 min, 100 μl HCl (0.1 M) were added to stop the enzymatic reaction, and then the absorbance at 560 nm was measured (E_s). To standardize the LDH activity before exposure to H_2O_2, myocytes were lysed by treatment with Tween 20 in EBSS (0.2% wt/vol for 1 h), and the LDH activity in Tween 20 solution (E_l) was determined. The background LDH activity in the supernatant of myocyte cultures without H_2O_2 was also measured (E_c). The relative LDH activity of each sample was calculated using the following formula: % LDH release $= 100 \times (E_s - E_c)/(E_l - E_c)$. E_l and E_c were measured under each set of experimental conditions.

Materials. Kodak X-Omat RR film was from Kodak (Rochester, NY). The automated analyzer used for the measurement of nitrite ion concentrations, TCI-NOX, was from Tokyo Chemical Industry (Tokyo, Japan). The spectrophotometer for the measurement of GPX activity, UV-PC 3700, was from Shimadsu (Tokyo, Japan). The culture medium was from GIBCO (Grand Island, NY). IL-1β was from Genzyme (Boston, MA). The cDNA probe for iNOS was a gift from Dr. Jean-Luc Balligand (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). The monoclonal antibody against mouse macrophage iNOS was purchased from Transduction Laboratories (Lexington, KY). The enhanced chemiluminescence system and the cGMP ELISA kit were from Amersham (Buckinghamshire, UK). The LDH assay kit, MTX-LDH, was from Kyokuto Pharmaceutical Industries (Tokyo, Japan). All other chemicals were purchased from Sigma (St. Louis, MO).

Statistics. At least five independent myocyte preparations were evaluated under each set of experimental conditions. For each set of experimental conditions, there were at least six dishes or wells. Data were expressed as the means ± SE of values from independent experiments. The statistical significance of differences between group means was analyzed by analysis of variance and Scheffé's *F*-test using STAT VIEW II (1988, Abacus Concepts, Berkeley, CA). A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Induction of iNOS and production of nitrite by cardiac myocytes. In cardiac myocytes stimulated with an inflammatory cytokine, IL-1β, the iNOS mRNA signal increased in a concentration-dependent manner, but iNOS mRNA levels in nonstimulated myocytes did not increase (Fig. 1*A*). Cardiac myocytes treated with IL-1β expressed an iNOS protein signal at 130 kDa, but myocytes not treated with IL-1β did not (Fig. 1*B*). Expression of iNOS protein signal was the strongest after 24-h incubation with IL-1β (data not shown).

In the absence of IL-1β, L-Arg failed to increase NO production (Fig. 2*A*). Nitrite production by myocytes in the presence of IL-1β was enhanced in a concentration-dependent manner by L-Arg. Inhibition of iNOS with L-NAME markedly reduced nitrite production in the

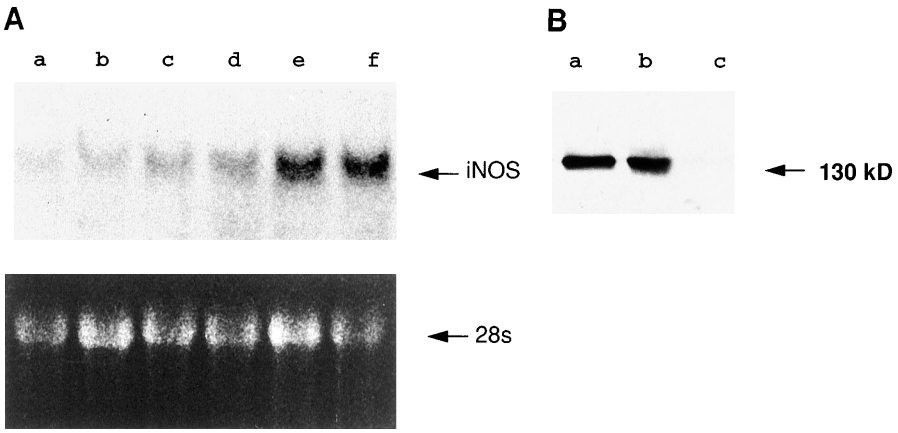

Fig. 1. Expression of inducible nitric oxide synthase (iNOS) mRNA and iNOS protein in cardiac myocytes. After incubation with interleukin-1β (IL-1β; 10 ng/ml) for 24 h, total mRNA or total protein was extracted from cultured cardiac myocytes and then analyzed as described in METHODS. *A*, *top*: Northern blot analysis for iNOS mRNA using cDNA probes from iNOS mRNA from rat vascular smooth muscle cells. Myocytes had been incubated with IL-1β at a concentration of (in ng/ml) 0 (*lane a*), 0.01 (*lane b*), 0.1 (*lane c*), 1.0 (*lane d*), 5 (*lane e*), or 10 (*lane f*). *Bottom*: ribosomal (28S) RNA by ethidium bromide fluorescence. *B*: Western blot analysis for iNOS protein using antibody to mouse macrophage iNOS protein. *Lane a*, mouse macrophage iNOS protein; *lane b*, extract from myocytes treated with IL-1β (10 ng/ml); *lane c*, extract from control myocytes not treated with IL-1β.

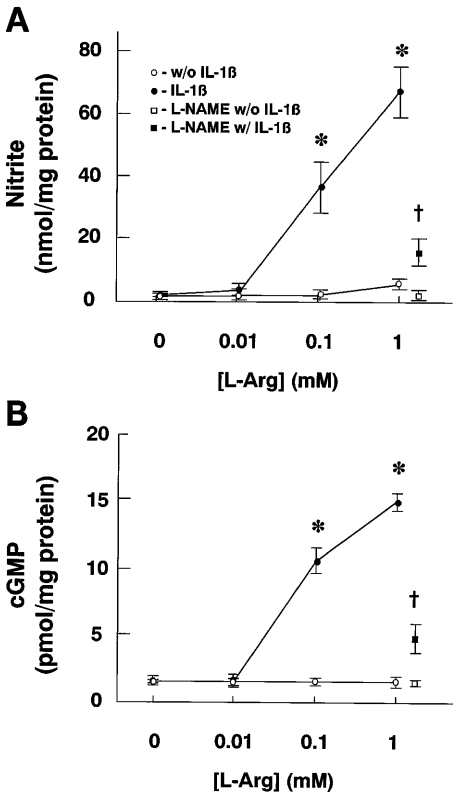

Fig. 2. Effects of IL-1β, L-arginine (L-Arg), and N^{ω}-nitro-L-arginine methyl ester (L-NAME) on nitric oxide production and cGMP levels in rat cardiac myocytes. Nitrite production in culture medium (*A*) and cGMP content of myocytes (*B*) were determined after 24 h of culture with indicated concentrations of L-Arg in presence and absence of IL-1β (10 ng/ml). In some experiments, L-NAME (1 mM) was added to cultures with 1 mM L-Arg. Each data point represents mean ± SE values from 6 batches of myocyte cultures. $*P < 0.05$ vs. data from IL-1β-treated cells at 0 mM L-Arg. $\dagger P < 0.05$ vs. IL-1β-treated cells at 0 mM L-NAME and 1 mM L-Arg by analysis of variance followed by Scheffé's test.

presence of L-Arg and IL-1β. Because of the effects on the pH of the culture medium, no more than 1 mM L-NAME could be added.

In the absence of IL-1β, L-Arg failed to increase the cGMP content of myocytes (Fig. 2*B*). In the presence of IL-1β, L-Arg significantly increased the cGMP content of myocytes (Fig. 2*B*). L-NAME attenuated the increase of cGMP levels by L-Arg in the presence of IL-1β.

Effects of NO production on GPX activity of cardiac myocytes. IL-1β alone did not affect the cytosolic GPX activity of cardiac myocytes in the absence of L-Arg (Fig. 3*A*). Concentrations of L-Arg that increased NO production markedly decreased the GPX activity of IL-1β-treated cardiac myocytes. L-NAME, at a concentration that significantly attenuated NO production by cardiac iNOS, reversed the L-Arg-induced attenuation of GPX activity. Similar to the effects on NO production, neither L-Arg nor L-Arg plus L-NAME affected GPX activity in the absence of IL-1β.

The exogenous NO donor, SNAP, was able to mimic the effects of endogenous NO production on GPX activity (Fig. 3*B*). GPX activity was decreased by SNAP in a concentration-dependent manner and was attenuated by 69% by 5 mM SNAP. *N*-acetyl-penicillamine (5 mM), a denitroso derivative of SNAP, did not change the GPX activity of myocytes.

To examine the involvement of the NO-guanylate cyclase pathway in the attenuation of GPX activity by NO, myocytes were treated with 8-bromo-cGMP (8-BrcGMP, 0.5 mM for 1 h), a cGMP analog. 8-BrcGMP did not affect the activity of GPX in myocytes (153.2 ± 10.9 vs. 154.7 ± 14.2 mU/mg protein, P = NS), indicating that cGMP levels did not affect GPX activity directly.

Effect of NO production on LDH release from cardiac myocytes. Oxidative injury of myocytes treated with H_2O_2 resulted in release of LDH into the culture medium (Fig. 4*A*). Neither treatment with L-Arg nor

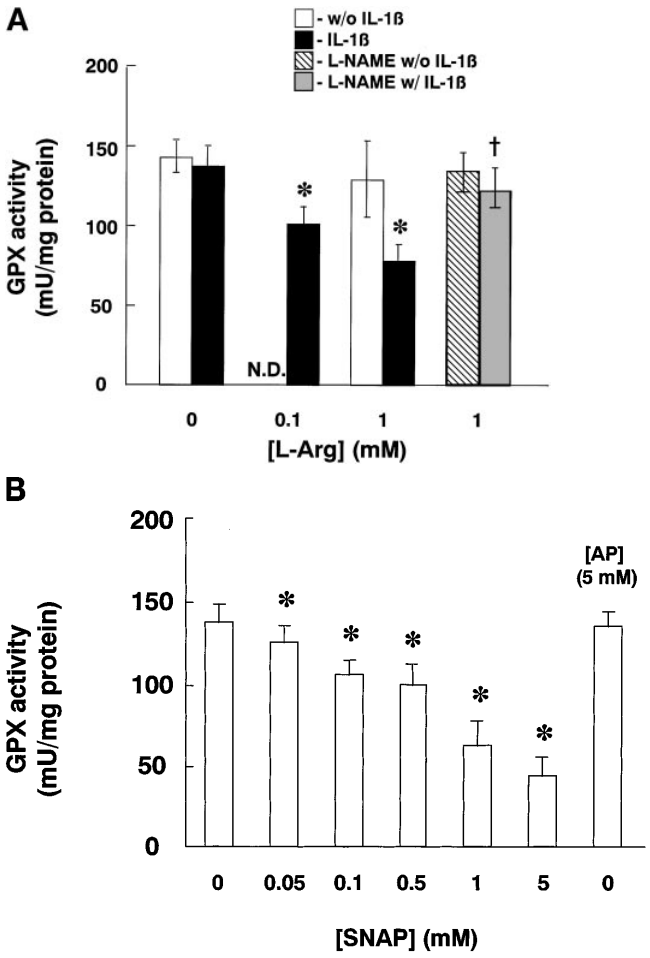

Fig. 3. Effects of IL-1β, L-Arg, L-NAME, and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on glutathione peroxidase (GPX) activity of rat cardiac myocytes. *A*: myocytes were cultured with indicated concentrations of L-Arg in presence or absence of IL-1β (10 ng/ml) for 24 h before being assayed for GPX activity. In some experiments, L-NAME (1 mM) was added to cultures with 1 mM L-Arg. Data are represented by mean ± SE values from 6 batches of myocyte cultures. ND, not determined. $*P < 0.05$ vs. data from IL-1β-treated cells at 0 mM L-Arg. $\dagger P < 0.05$ vs. IL-1β-treated cells at 0 mM L-NAME and 1 mM L-Arg by analysis of variance followed by Scheffé's test. *B*: myocytes were treated with indicated concentrations of SNAP or with *N*-acetyl-penicillamine (AP; 5 mM) for 1 h (instead of IL-1β for 24 h) before measurement of GPX activity. Data are represented by mean ± SE values from 6 batches of myocyte cultures. $*P < 0.05$ vs. data from cells at 0 mM SNAP by analysis of variance followed by Scheffé's test.

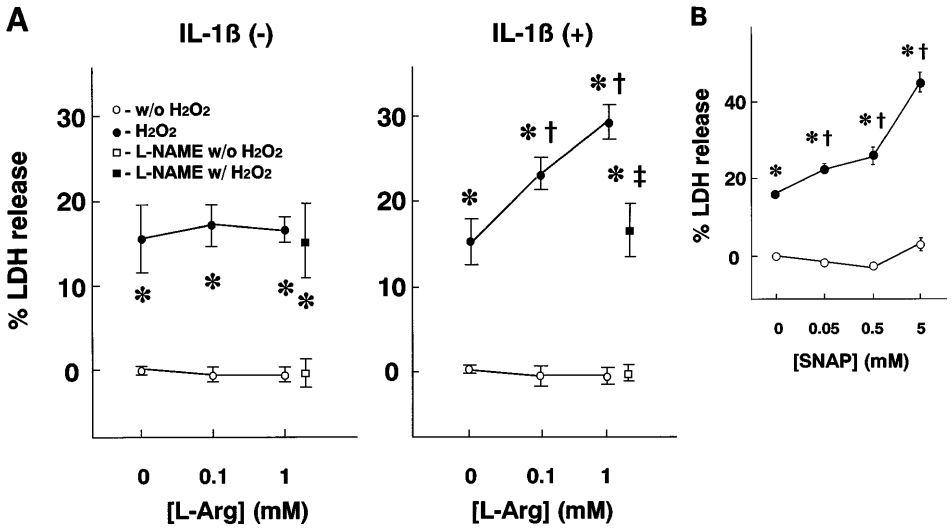

Fig. 4. Effects of L-Arg, L-NAME, and SNAP on H_2O_2-induced lactate dehydrogenase (LDH) release from rat cardiac myocytes. *A*: myocytes were treated for 24 h with indicated concentrations of L-Arg in presence or absence of IL-1β (10 ng/ml). In some experiments, L-NAME (1 mM) was added to cultures with 1 mM L-Arg. Then, myocytes were treated with H_2O_2 (0.1 mM) for 1 h before determination of LDH activity in culture medium. Each data point represents mean ± SE of values from 6 batches of myocyte cultures. *$P < 0.05$ vs. without H_2O_2. †$P < 0.05$ vs. H_2O_2-treated cells at 0 mM L-Arg. ‡$P < 0.05$ vs. 0 mM L-NAME at 1 mM L-Arg by analysis of variance followed by Scheffé's test. *B*: myocytes were treated for 1 h with indicated concentrations of SNAP before treatment with H_2O_2 (0.1 mM for 1 h). LDH activity in culture medium was determined thereafter. Each data point represents mean ± SE of values from 6 batches of myocyte cultures. *$P < 0.05$ vs. without H_2O_2. †$P < 0.05$ vs. 0 mM SNAP plus H_2O_2 by analysis of variance followed by Scheffé's test.

treatment with L-Arg plus L-NAME elicited release of LDH, and neither treatment altered the response of myocytes to H_2O_2. Also, treatment with IL-1β plus L-Arg did not elicit release of LDH in the absence of H_2O_2.

However, in the presence of IL-1β, H_2O_2-induced LDH release from myocytes was augmented in a concentration-dependent manner by L-Arg, parallel with NO production. In the presence of IL-1β, L-NAME prevented the L-Arg-induced augmentation of H_2O_2-induced LDH release. IL-1β, L-Arg, or L-NAME plus L-Arg did not affect the LDH activity in the medium of cells lysed with Tween 20 (data not shown), suggesting that the myocyte LDH activity was not directly modulated by these reagents.

The H_2O_2-induced release of LDH from myocytes was augmented in a concentration-dependent manner by prior treatment with SNAP (Fig. 4*B*). SNAP did not increase the release of LDH in the absence of H_2O_2. *N*-acetyl-penicillamine did not exacerbate the damage induced by H_2O_2 (14.9 ± 0.9 vs. 15.8 ± 1.3%, P = NS). The lower concentration of H_2O_2 (0.01 mM for 1 h) did not exert significant LDH release without SNAP. However, when myocytes were treated with SNAP (5 mM for 1 h) before H_2O_2, LDH release markedly increased compared with those treated with H_2O_2 without SNAP (0.3 ± 0.5 vs. 7.2 ± 2.5%, $P < 0.05$). Thus GPX inhibition by NO exerts myocardial injury at milder oxidative stress, which does not cause myocardial injury by itself. To examine whether or not an exogenous antioxidant can salvage myocardial injury induced by the inhibition of GPX acitivity by NO, we added *N*-acetyl-cysteine to myocyte cultures. *N*-Acetyl-cysteine (5 mM) partly reversed injury of SNAP-treated myocytes after H_2O_2 treatment (42.9 ± 2.4 vs. 23.5 vs. 1.5%, $P < 0.05$).

Effects of antisense ODN for GPX mRNA on GPX activity and H_2O_2-elicited myocardial injury. The activity of GPX in myocytes was decreased in a concentration-dependent manner by incubation with antisense ODN for 2 days (Fig. 5). Shorter period of incubation with antisense ODN did not decrease GPX activity significantly (data not shown). Sense ODN and scrambled ODN failed to decrease GPX activity in myocytes.

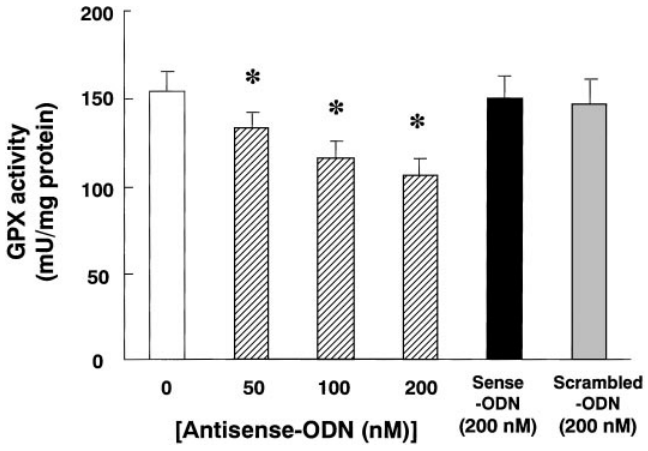

Fig. 5. Effect of incubation with oligodeoxyribonucleotides on GPX activity of rat cardiac myocytes. Myocytes were incubated for 2 days with indicated concentrations of antisense (antisense ODN), sense (sense ODN), or scrambled (scrambled ODN) oligodeoxyribonucleotides for rat GPX cDNA before measurement of GPX activity. Each data point represents mean ± SE of values from 6 batches of myocyte cultures. *$P < 0.05$ vs. 0 nM antisense ODN by analysis of variance followed by Scheffé's test.

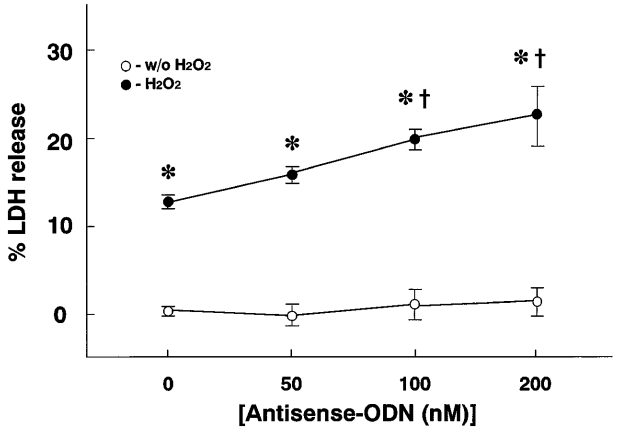

Fig. 6. Effect of antisense ODN on H_2O_2-induced release of LDH from rat cardiac myocytes. Myocytes were incubated for 2 days with indicated concentrations of antisense ODN for rat GPX cDNA. Myocytes were treated with H_2O_2 (0.1 mM) for 1 h before determination of LDH activity in culture medium. Each data point represents mean ± SE values from 6 batches of myocyte cultures. *$P < 0.05$ vs. without H_2O_2. †$P < 0.05$ vs. H_2O_2-treated cells at 0 nM antisense ODN by analysis of variance followed by Scheffé's test.

After it was confirmed that antisense ODN for GPX mRNA decreases GPX activity of cardiac myocytes, myocytes were treated with H_2O_2 after incubation with antisense ODN. LDH release from myocytes after H_2O_2 treatment was augmented by prior incubation with antisense ODN (Fig. 6). Antisense ODN did not induce LDH release from myocytes in the absence of H_2O_2. Neither sense ODN nor scrambled ODN augmented H_2O_2-induced LDH release. None of the ODNs altered activity in the culture medium of myocytes lysed with Tween 20 (data not shown).

DISCUSSION

IL-1β induces the cardiac myocytes to produce NO in the presence of L-Arg, and production is inhibited by L-NAME. Also, NO production paralleled cGMP production and iNOS expression as detected by Northern blotting and Western blotting, indicating that cardiac iNOS produces NO. In this study, the production of NO by cardiac myocytes in the presence of L-Arg was not detected in the absence of IL-1β, despite constitutive expression of eNOS protein in cardiac myocytes (3). The absence of detectable levels of NO may have been due to the low sensitivity of the Griess reagent reaction, but without IL-1β, neither L-Arg nor L-NAME affected GPX activity, cGMP level, or myocardial injury after H_2O_2 treatment. Thus changes in cardiac iNOS appeared to account for the changes observed in these indicators. Also, the estimated cytosolic L-Arg concentration (0.1–0.8 mM; Ref. 15) is equivalent to the L-Arg concentration used in the present study, indicating that cardiac iNOS produces NO in vivo utilizing L-Arg as a substrate.

Endogenous as well as exogenous NO attenuated the activity of GPX in cardiac myocytes. We have reported that SNAP inactivates bovine GPX in vitro and GPX in U-937 cells. The NO produced by iNOS also inactivates GPX in a macrophage cell line (2), indicating that such inactivation of GPX is a general effect of NO in cellular systems. Because GPX contains cysteine residues and a seleno-cysteine residue in its catalytic center (34), modification of the enzyme activity through *S*- or Se-nitrosylation (39) might be a mechanism of inactivation.

Neither exogenous nor endogenous NO appeared to injure myocytes, but both treatments augmented the H_2O_2-induced LDH release. Because NO did not affect the levels of LDH in myocytes before H_2O_2 treatment, these results indicate that NO increases the vulnerability of myocytes to H_2O_2. Oxidative stress is important in the pathogenesis of various heart diseases due to the near total dependence of this organ on aerobic metabolism. Elimination of H_2O_2 is critical to protect heart tissue against oxidative stress, because superoxide, a relatively inactive oxygen free radical at a biological pH, is converted to H_2O_2 by SOD, and in the presence of cardiac myocytes, H_2O_2 forms hydroxyl radical (·OH), one of the most toxic oxygen free radicals, via Fenton reaction (23). Thus dysfunction of the H_2O_2 elimination system could result in severe tissue damage from oxidative stress. GPX appears to act as a key enzyme degrading H_2O_2 in the cytosol of cardiac myocytes, because myocytes do not have peroxisomes that contain catalase. The amount of GPX in the heart is comparable to the amount in the liver (31). Results of the present study demonstrate that the attenuation of GPX activity could account for the increase in H_2O_2-elicited myocardial cell damage.

Various extracellular stimuli modify content or activity of cardiac antioxidant enzymes, not only GPX, but also catalase (8) and SOD (18, 42). IL-1β, which used to induce iNOS, also increased Mn-SOD content in our experiment (data not shown). Therefore, there is a possibility that the change in antioxidant enzymes other than GPX may modulate myocardial injury after exposure to H_2O_2 when pretreated with IL-1β. However, IL-1β in the presence of L-Arg increased myocardial injury after H_2O_2 treatment in the present study, suggesting that induction or activation of other antioxidant enzyme seems to be insufficient to compensate the inhibition of GPX activity by NO. We also examined that NO exclusively attenuated GPX activity, but not catalase or SOD activity in our previous paper (2), and that the specific inhibition of GPX activity by SNAP or by antisense ODN to GPX mRNA increased myocyte injury after H_2O_2 treatment in this experiment. Therefore, inactivation of GPX by NO seems to play a significant role in the augmentation of myocardial injury after H_2O_2 treatment.

In the present study, induction of cardiac iNOS per se did not appear to induce myocyte injury. However, it is reasonable to assume that the myocardium is exposed to oxidative stress in many pathological conditions. An increase in oxidative stress has been observed in heart diseases such as heart failure (16, 33) and myocardial ischemia (27, 45). In addition to attenuating GPX activity, NO might directly produce a toxic oxygen metabolite, peroxynitrite ($ONOO^-$, Ref. 6). $ONOO^-$ may cause cellular injury through the production of

·OH (6) and the peroxidation of membrane lipids (17, 36). $ONOO^-$ formation has direct deleterious effects to isolated cardiac myocytes (21) and the isolated heart (37). Ishiyama et al. (22) reported that inhibition of cardiac iNOS by aminoguanidine, a relatively selective inhibitor of iNOS, markedly inhibited the progression of myocardial damage in rat autoimmune myocarditis (22). The mechanism by which NO promotes rat autoimmune myocarditis is thought to be via formation of $ONOO^-$. In the present study, the absence of leukocytes as a source of oxygen free radicals (26) could explain the lack of toxic effects of NO alone. Thus in vivo induction of iNOS in heart tissue might be toxic to myocardium.

Antisense ODN for GPX mRNA is the only specific inhibitor of this enzyme available for cultured cells. Antisense ODN for GPX mRNA markedly attenuated the activity of GPX and significantly increased myocardial cell injury after H_2O_2 treatment. Because the sense and scrambled ODN molecules did not alter GPX activity, nonspecific effects of ODN were considered minimal. However, attenuation of GPX activity by antisense ODN was not complete (30% at 200 nM). Antisense ODN for Mn-SOD also exhibits partial inhibition in cultured neonatal rat cardiac myocytes (42). Thus it may be difficult to completely inhibit the constitutive antioxidant enzymes in cultured neonatal rat cardiac myocytes by means of antisense ODN. Interestingly, Yoshida and Maulik (43) recently reported that myocardial damage after ischemia-reperfusion increased in knockout mice heterozygous for the GPX gene whose GPX activity in myocardium was partially attenuated, consistent with the hypothesis that GPX is important in protecting myocardium against oxidative stress. We were surprised to find that inactivation of GPX by iNOS, SNAP, or antisense ODN did not produce myocardial cellular injury unless the myocytes were also subjected to oxidative stress. Although oxidative stress alone was sufficient to injure the myocytes, the levels of injury were exacerbated by events that increased the availability of NO. Because such treatments did not affect the activity of LDH directly, it appears that the inhibition of GPX by NO increased the susceptibility of myocytes to H_2O_2.

In summary, treatments that increased the expression of iNOS in cultured rat neonatal cardiac myocytes reduced the activity of GPX, an antioxidant enzyme, and increased the damage to myocytes after H_2O_2 treatment. Treatment with an exogenous NO donor was able to mimic these effects. These results suggest that increased levels of NO resulting from the induction of iNOS exacerbate myocardial damage due to oxidative stress. The selective inhibition of iNOS may be a novel way of slowing the progression of heart disease associated with oxidative stress.

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