We investigated the effects of precisely controlled mechanical strain on nitric-oxide synthase activity in cultured neonatal rat cardiac myocytes. Incubation of cardiac myocytes for 24 h with 4 ng/ml interleukin-1β and 100 units/ml interferon-γ stimulated an increase in nitric oxide production, inducible nitric-oxide synthase (iNOS) mRNA, and iNOS protein. Mechanical strain suppressed nitric oxide production, iNOS mRNA, and iNOS protein stimulated by cytokines in an amplitude-dependent manner. Losartan (1 μM), an angiotensin II type 1 receptor antagonist, weakly inhibited the effect of strain, suggesting that paracrine angiotensin II is not the mediator of the strain effect. In addition, cycloheximide (10 μM), a protein synthesis inhibitor, inhibited the effect of strain by 46%. Transforming growth factor-β (1 ng/ml) suppressed iNOS mRNA expression, but anti-transforming growth factor-β antibody (30 μg/ml) did not block the effect of strain. In contrast, staurosporine (100 nM; a nonspecific protein kinase inhibitor), calphostin C (1 μM; a selective protein kinase C inhibitor), and pretreatment with phorbol 12-myristate 13-acetate abolished the effect of strain. Genistein (100 μM), a tyrosine kinase inhibitor, partially inhibited the effect of strain. Thus, cyclic mechanical deformation suppresses cytokine-induced iNOS expression in cardiac myocytes, and this effect is mediated at least partially via activation of protein kinase C.

Nitric oxide (NO)1 plays an important role as an intercellular messenger in most or all mammalian tissues (1–4). Three genes encode nitric-oxide synthase of two biochemical types. Type I neuronal and type III endothelial nitric-oxide synthases are constitutive but dormant until activated primarily by Ca2+ transients that sustain the binding of calmodulin. A third NOS, inducible nitric-oxide synthase (iNOS, or type II nitric-oxide synthase), is expressed only after transcriptional induction (5).

*This work was supported in part by a grant-in-aid from the American Heart Association and NHLBI Grants HL-54759 and HL-52320 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported in part by grants from the Uehara Memorial Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Japan Foundation of Cardiovascular Research.

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1The abbreviations used are: NO, nitric oxide; iNOS, inducible nitric-oxide synthase; IL-1β, interleukin-1β; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; FMA, phorbol 12-myristate 13-acetate; NRVM, neonatal rat ventricular myocytes; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
modified Eagle’s medium (DMEM; BioWhittaker, Inc., Walkersville, MD) containing 7% fetal calf serum (FCS; Life Technologies, Inc.), 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.).

**Mechanical Strain Device and Preparation of Cells—**Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured, an approach that produces controlled cellular strain as well as visualization of cells. The device used in this study provides a nearly homogeneous and uniform biaxial strain profile, i.e., strains that are equal at all locations on the membrane and in all directions. This approach eliminates locations on the substrate that have very high strains (20–30%) in one direction. We have previously measured membrane strains with high-resolution video (31); the cans used for this study gave strains of 1, 4, 9, and 14%.

NRVM were plated on a fibronectin-coated (2 μg/ml) membrane dish at a density of 3,000,000 cells/dish in 13 ml of DMEM containing 7% FCS and incubated for 48 h. Approximate cell confluence was 85–90%. NRVM were then made quiescent by washing four times with 10 ml of Hanks’ balanced salt solution and incubating with 10 ml of DMEM containing 1% insulin/transferrin/seleminium media supplement (ITS, Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin. All experiments were performed on NRVM that had been serum-starved for 24 h. Mechanical strain was then applied at a specified constant frequency and amplitude, and control dishes received no mechanical strain.

**Measurement of Nitrite Concentration—**The formation of nitrite was used as an indicator of NO release by NRVM. Nitrite accumulation was measured by the Griess reaction as described previously (32). Briefly, the medium was centrifuged at 3000 rpm for 10 min at 4 °C to remove cellular debris. The nitrite content in the supernatant was measured by combining 150 μl of medium with 900 μl of Griess reagent (0.75% sulfanilamide in 0.5 N HCl and 0.057% naphthylethylenediamine dihydrochloride), and the concentration of the resultant chromophore was determined spectrophotometrically at 543 nm. Nitrite accumulation was calculated from known concentrations of sodium nitrite within the linear range of the assay (0.5–20 μM).

**Western Blot of iNOS Protein—**NRVM were lysed directly in each dish by application of 2% sample buffer containing 250 mM Tris-HCl (pH 6.8), 10% glycerol, 4% SDS, and 2% β-mercaptoethanol, and the mixture was boiled for 5 min. The lysate was centrifuged, and the supernatant was collected. Total protein concentration was measured by the Bradford method (Bio-Rad), and equal quantities of total protein were loaded on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane in 25 mM Tris base (pH 8.5), 0.2 M glycine, and 20% methanol. The nitrocellulose membrane was blocked with 5% nonfat dried milk in Tris-buffered saline washing buffer containing 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 for 2 h. For the detection of iNOS, the membrane was incubated with 1:1000 diluted mouse monoclonal anti-iNOS antibody (Transduction Laboratories, CA). For Northern blotting, 15 and the Klenow fragment of DNA polymerase (Stratagene, La Jolla, CA). For Northern blotting, 15 μg of total RNA was loaded on a 1.0% agarose gel, UV-cross-linked with a UV Stratalinker (Stratagene), and UV-cross-linked with a UV Stratalinker (Stratagene). The probe was hybridized with QuikHyb solution (Stratagene) at 68 °C for 1 h. The washed membrane was exposed to x-ray film overnight at −80 °C. Normalization of RNA for equal loading was carried out by rehybridizing the blots with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (CLONTECH, Palo Alto, CA).

**Statistical Analysis—**Data are expressed as the means ± S.E. of four samples, which represented at least three separate experiments. Differences were analyzed by one-way analysis of variance combined with Sheffe’s test, and p values < 0.05 were considered to be statistically significant.

**RESULTS**

**Effects of Cytokines and Mechanical Strain on Nitrite Production by NRVM—**We first investigated the effect of the combination of IL-1β and IFN-γ on NO production by cultured cardiac myocytes. As shown in Fig. 1, treatment of NRVM with IL-1β (4 ng/ml) and IFN-γ (100 units/ml) caused a marked accumulation of nitrite in the culture medium in a time-dependent manner. However, surprisingly, this cytokine-induced nitrite accumulation in NRVM was significantly suppressed by 9% cyclic mechanical strain at 1 Hz. 9% cyclic mechanical strain by itself did not affect the basal levels of nitrite production by NRVM.

**Effects of Mechanical Strain on iNOS Protein and mRNA Accumulations—**We next investigated whether mechanical strain suppressed increases in iNOS protein and mRNA levels in cytokine-stimulated NRVM. The expression of iNOS protein was analyzed by immunoblotting with anti-iNOS antibody. No immunoreactive iNOS was detected in unstimulated and stretched NRVM (Fig. 2). The iNOS protein band with a molecular mass of 130 kDa appeared after exposure to the combination of IL-1β and IFN-γ in a time-dependent manner, and these iNOS protein accumulations were markedly suppressed by 9% cyclic mechanical strain at 1 Hz (Fig. 2). Furthermore, as shown in Fig. 3, unstimulated and stretched NRVM expressed no detectable iNOS mRNA, whereas exposure to the combination of IL-1β and IFN-γ clearly induced iNOS mRNA accumulations in a time-dependent manner. 9% cyclic mechanical strain at 1 Hz suppressed the cytokine-stimulated increase in iNOS mRNA accumulation. In seven separate experiments, strain suppressed iNOS mRNA by 61 ± 4% (p < 0.001). In addition, when NRVM were subjected to cyclic strains of 1, 4, 9, and 14% at 1 Hz for 8 h, the effect of mechanical strain on iNOS mRNA expression was amplitude-dependent (Fig. 4). In these studies, no morphologic changes in cardiac myocytes were detected following strains of 1–14%.

**Effects of Angiotensin II Type 1 Receptor Antagonism on Suppression of iNOS mRNA Expression by Mechanical Strain—**Mechanical strain can cause release of angiotensin II from cardiac myocytes, which acts as a mediator of the stretch-induced hypertrophic response via the angiotensin II type 1 receptor (23). To explore the mechanism of the suppressive effects of mechanical strain on NO synthesis, we investigated whether the suppressive effect of cyclic mechanical strain on iNOS mRNA expression in cytokine-stimulated NRVM is angiotensin II-dependent. Cytokine-stimulated NRVM were stretched using 9% cyclic strain at 1 Hz for 8 h in the presence or absence of angiotensin II type 1 receptor antagonist. As shown in Fig. 5, losartan (1 μM), an angiotensin II type 1 receptor antagonist, weakly inhibited (11% reduction by den-
Effects of mechanical strain on iNOS protein expression. NRVM were plated on 2 μg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation for 24 h, myocytes were exposed to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml) for 24 h. Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using mouse anti-iNOS antibody. The molecular mass of iNOS protein is 130 kDa. Data are representative of two independent experiments with nearly identical results.

Effects of TGF-β and anti-TGF-β Antibody on Suppression of iNOS mRNA Expression by Mechanical Strain—TGF-β, released by cardiac myocytes and acting in an autocrine and/or paracrine manner, may participate in myocardial remodeling by hypertrophic stimuli (35). Induction of iNOS is inhibited in cardiac myocytes by TGF-β (34, 36, 37). We investigated whether TGF-β is involved in the suppressive effect of mechanical strain in cytokine-stimulated NRVM. In addition, anti-TGF-β antibody (30 μg/ml) slightly augmented iNOS mRNA expression and did not abolish the suppressive effect of 9% cyclic mechanical strain (1 Hz) on iNOS mRNA expression in cytokine-stimulated NRVM (5%). Neither TGF-β nor anti-TGF-β antibody by themselves induced iNOS mRNA expression in NRVM. This experiment suggests that TGF-β secreted from cardiac myocytes does not play an important role in the suppressive effect of mechanical strain on iNOS mRNA expression.

Effects of angiotensin II type 1 receptor antagonism on suppression of iNOS mRNA expression by mechanical strain. NRVM were plated on 2 μg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation for 24 h, myocytes were exposed for 8 h to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml) in the presence or absence of losartan (1 μM). Losartan was applied to the myocytes 30 min before mechanical strain. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.

Effects of cycloheximide on suppression of iNOS mRNA expression by mechanical strain. NRVM were plated on 2 μg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation for 24 h, myocytes were exposed for 8 h to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml) in the presence or absence of cycloheximide (10 μM). Cycloheximide was applied to the myocytes 30 min before mechanical strain. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.

Effects of Cycloheximide on Suppression of iNOS mRNA Expression by Mechanical Strain—Cycloheximide (10 μM), a protein synthesis inhibitor, for 8 h slightly suppressed iNOS mRNA in cytokine-stimulated NRVM (Fig. 6). However, cycloheximide modestly inhibited (46%) the effect of 9% cyclic mechanical strain. This experiment supported the premise that cyclic mechanical strain directly suppresses iNOS mRNA expression without new protein synthesis.

Protein Kinase C and Tyrosine Kinase Inhibition—To examine the role of protein kinase C and tyrosine kinases in the suppression of iNOS mRNA expression by mechanical strain, we tested the effect of protein kinase C and tyrosine kinase inhibitors (15–19). As shown in Fig. 8, addition of staurospo-
Strain Suppresses iNOS Expression

Shear stress and cyclic mechanical strain represent important components of the mechanical environment that regulate gene expression in endothelium, vascular smooth muscle, and myocardium. In bovine aortic endothelial cells, cyclic mechanical strain augments endothelial NOS expression and NO production (28). In human aortic endothelial cells, pulsatile stretch increases both endothelial NOS protein and mRNA expression (29). Wagner et al. (38) reported that shear stress and cyclic strain induce the expression of heme oxygenase-1, the enzyme that forms carbon monoxide, but not of iNOS mRNA in cultured vascular smooth muscle cells. In the present study, mechanical strain did not induce NO production and iNOS expression in cardiac myocytes, whereas mechanical strain suppressed NO production and iNOS expression in cytokine-stimulated myocytes. These findings suggest that mechanical strain may play an important role in iNOS expression in immunological and inflammatory conditions, including postcardiac transplantation, cardiomyopathy, myocarditis, and ischemia-reperfusion injury.

Although cardiac mechanical overload is known to alter the expression of several cardiac-specific genes, it has not yet been established whether deformation acts directly or via local paracrine and autocrine factors liberated in response to hemodynamic load. In particular, the local renin-angiotensin system may play an important role in the adaptation of the heart to pressure and volume overload (39, 40). Angiotensin II secreted from cardiac myocytes is an important mediator of the strain-induced hypertrophic response, but factors other than angiotensin II may be involved in cardiac hypertrophy induced by mechanical strain (23, 41–43). In the present study, losartan weakly inhibited the suppressive effect of mechanical strain on iNOS mRNA expression in cytokine-stimulated cardiac myocytes. Thus, it is likely that factors other than angiotensin II are also involved in the response to mechanical strain.

TGF-β is secreted from cardiac myocytes by hypertrophic stimuli (35). TGF-β, which stimulates iNOS expression in 3T3 fibroblasts (44), diminishes iNOS expression in response to cytokines in cardiac myocytes (34, 36, 37) as well as microvascular endothelial cells (45) and vascular smooth muscle cells (46). Perrella et al. (47) demonstrated that the ability of TGF-β to suppress iNOS promoter/enhancer activity occurs through a site(s) other than the NF-κB motif in vascular smooth muscle cells. In the present study, anti-TGF-β antibody did not alter the suppressive effect of mechanical strain on iNOS mRNA expression in cytokine-stimulated cardiac myocytes. It is therefore unlikely that TGF-β is the initial mediator for the suppressive effects of mechanical strain on iNOS mRNA expression. Furthermore, cycloheximide partially inhibited the effect of mechanical strain. These findings suggest that mechanical strain directly acts on iNOS gene expression in cardiac myocytes without new paracrine protein synthesis.

Protein kinase C pathways activated by mechanical strain play important roles in hypertrophy and gene expression in cardiac myocytes (19). Stretch-induced c-fos expression is inhibited by both protein kinase C inhibitors and down-regulation of protein kinase C (15, 19). Recently, Obadia et al. (48) reported that protection against myocardial ischemia in rats could be induced by stretch, likely through activation of protein kinase C. On the other hand, many factors appear to be involved in the complex regulation of iNOS induction in response to cytokines (49–53). In the present study, protein kinase C inhibitors and pretreatment with PMA abolished the suppressive effects of mechanical strain on iNOS mRNA expression. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.

**DISCUSSION**

**FIG. 7.** Effects of TGF-β and anti-TGF-β antibody on suppression of iNOS mRNA expression by mechanical strain. NRVM were plated on 2 µg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation for 24 h, myocytes were exposed for 8 h to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml) in the presence or absence of TGF-β (1 ng/ml), anti-TGF-β antibody (Ab; 30 µg/ml), or TGF-β + anti-TGF-β antibody. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.

**FIG. 8.** Effects of protein kinase C and tyrosine kinase inhibitors on suppression of iNOS mRNA expression by mechanical strain. NRVM were plated on 2 µg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation for 24 h, myocytes were exposed for 8 h to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml) in the presence or absence of staurosporine (100 nM), calphostin C (1 µM), or genistein (100 µM). These inhibitors were applied to the myocytes 30 min before mechanical strain. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.

**FIG. 9.** Effects of pretreatment with PMA on suppression of iNOS mRNA expression by mechanical strain. NRVM were plated on 2 µg/ml fibronectin in DMEM containing 7% FCS for 48 h. After serum deprivation and pretreatment with or without PMA (1 µM) for 24 h, myocytes were exposed for 8 h to 0 or 9% cyclic mechanical strain (1 Hz) with or without IL-1β (4 ng/ml) + IFN-γ (100 units/ml). Total RNA was isolated and analyzed by Northern blotting with 32P-labeled iNOS (upper panel) and GAPDH (G3PDH; lower panel) cDNA probes. Data are representative of two independent experiments with nearly identical results.
sive effect of mechanical strain on iNOS expression in cytokine-stimulated cardiac myocytes, suggesting that this effect is mediated at least partially via activation of protein kinase C. In conclusion, cyclic mechanical strain suppresses cytokine-induced iNOS expression in cardiac myocytes, at least partially via activation of protein kinase C. This mechanism may play a role in the pathophysiology of myocardial infarction, post-cardiac transplantation, cardiomyopathy, myocarditis, and congestive heart failure. In addition, these data demonstrate that mechanical stimuli are potent regulators of the molecular response of myocytes to cytokines.

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J. Biol. Chem. 1998, 273:11862-11866.
doi: 10.1074/jbc.273.19.11862

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