Interleukin-1β Induces Cardiac Myocyte Growth but Inhibits Cardiac Fibroblast Proliferation in Culture

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Abstract

Interleukin-1 (IL-1), initially called "endogenous pyrogen," is primarily known as a mediator of inflammation. However, it also plays many other diverse physiologic roles including the stimulation and inhibition of both primary cells in culture and the interstitial and parenchymal cells of a number of organs including the heart. In the heart, IL-1 expression has traditionally been reported in situations where there is immunologic myocardial injury such as occurs during transplant rejection and congestive heart failure. For this reason, all of the effects of IL-1 have been presumed to be deleterious. Using a cell culture model which allows both the muscle cells (myocytes) and nonmuscle cells (fibroblasts) to be evaluated separately, we have found that IL-1 induces both cardiac myocyte hypertrophy and reinitiates myocyte DNA synthesis. In stark contrast, IL-1 exerts a potent antiproliferative effect on cardiac fibroblasts. To our knowledge this is the first report concerning the differential effects of IL-1 on myocardial cell growth in culture and, given the inducible expression of IL-1 by myocardial cells during stress, underscores the importance of investigating the complex nature of the intracardiac cell–cell interactions that occur in the heart. (*J. Clin. Invest.* 1995. 95:2555–2564.)
Key words: hypertrophy • interleukin-1 • cardiac myocyte • cardiac fibroblast

Introduction

Interleukin-1 (IL-1 as used in this text refers to IL-1β), also known as "endogenous pyrogen," is a member of a family of cell activators called cytokines. Although the focus of investigations on these proteins has largely been related to their activation of cells involved in immune and inflammatory responses, they can also have profound effects on nonimmune cells and organs. The heart is one such organ whose function can be profoundly affected during periods of increased cytokine production such as occurs with endotoxic shock, transplant rejection, and ischemia/reperfusion (1–5). Fortunately, the myocardial contractile depression associated with these inflammatory myocardial disease states is usually not due to an increase in cell death and is reversible when appropriate treatment is undertaken. In reports using infusion studies of both in situ and explanted hearts (Langendorff preparations), as well as short-term treatment of high density neonatal rat myocyte cultures, investigators have implicated IL-1 as one of the inflammatory mediators responsible for the decrease in contractility seen during septic shock (6–11).

It has become evident from recent investigations in both our laboratory and others that the cells which make up the heart (myocytes and nonmyocytes) are both the source and the target of many peptide growth factors and cytokines (12–16). In this regard, we have recently identified the cardiac fibroblasts as a source of IL-1 in a cell culture model of myocardial hypoxia (17). However, the effect of IL-1 on the growth of myocardial cells (both myocytes and fibroblasts) over time in culture has not been rigorously investigated. Specifically, the decrease in cardiac contractility in response to IL-1 described above is an acute response (minutes–hours) while the hypertrophic/proliferative effect of growth factors must be evaluated over a longer time course (hours–days). To explore the effects of IL-1 on myocardial cell growth and proliferation, we have used a cell culture model which allows both the muscle cells (myocytes) and nonmuscle cells (fibroblasts) to be evaluated separately (15).

Since cardiac myocytes rapidly lose their ability to divide under basal conditions both in vivo and in culture, their growth response to various stimuli involves primarily the hypertrophy of individual cells. Although there have been reports of myocyte hyperplasia in response to some growth factors in culture, most of these agents are also potent inducers of cardiac fibroblast proliferation. As such, the degree of myocyte proliferation in these mixed cell culture systems is often difficult, if not impossible, to determine. In contrast to cardiac myocytes, however, cardiac fibroblasts maintain the ability to divide and respond to growth stimuli predominantly through an increase in cell numbers, a response that has been noted in vivo (for review see reference 18) and reproduced in culture (15, 19–21).

As is true for most peptide growth factors/cytokines, the biological action of IL-1 is mediated through specific cell surface receptors, and two types of IL-1 receptors have been identified (IL-1R1 and IL-1R2).[1] Cellular activation appears to occur solely through IL-1R1, with IL-1R2 acting as a "decoy receptor" (22, 23). Stimulation of IL-1R1 has been shown to upregulate a variety of signaling pathways, among them cyclooxygenase/prostaglandins, inducible nitric oxide synthase/nitric oxide, and protein kinase C (PKC). The IL-1R also has been shown in a variety of cell types to express second messengers through protein tyrosine phosphorylation, even though it does not contain sequences typical of a protein tyrosine kinase (TK) domain (24–26).

As a result of the studies described in this report, we have found that IL-1 induces hypertrophy of cardiac myocytes and also reinitiates myocyte DNA synthesis. As indicated by the

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; IL-1R1 and IL-1R2, IL-1 receptor types 1 and 2; IL-1RA, IL-1–receptor antagonist; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PKC, protein kinase C; TK, tyrosine kinase.

absence of the induction of either the skeletal α-actin or β-myosin heavy chain mRNAs, however, IL-1 does not appear to stimulate the fetal/neonatal transcriptional program in the same way seen with α1-adrenergic and other growth factor stimulation (27, 28). At the same time, IL-1 also exerts a potent antiproliferative effect on cardiac fibroblasts. The myocyte growth effect appears to act through a TK signaling pathway in that it is inhibited by the inhibitor genistein. The growth effect we have seen with IL-1 is not common to all cytokines in that neither IL-6 nor γ-interferon (γIFN) was found to induce growth.

Methods

Materials. Minimum essential medium (MEM) with Hanks' salts was obtained from the Cell Culture Facility, University of California, San Francisco. Calf serum was obtained from Hyclone Labs (Logan, UT). Insulin was obtained from Eli Lilly & Co. (Indianapolis, IN). ^{14}C-phenylalanine (500 mCi/mmol), [^{3}H]thymidine (20 Ci/mmol), and [^{32}P]UTP (3,000 Ci/mmol) were obtained from DuPont/New England Nuclear (Boston, MA). The human *gro* cDNA was a kind gift of Dr. Ruth Sager (Dana-Farber Cancer Institute, Boston, MA). Recombinant mouse IL-1β, IL-6, γIFN, and rat *gro*β were obtained from Genzyme Corp. (Cambridge, MA). Rat IL-RA protein was the kind gift of Dr. Stephen Poole (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom). For RNAse protection, the RPA II kit from Ambion Inc. (Austin, TX) was used according to the manufacturers recommendations.

Cell culture. Primary cultures of neonatal rat cardiac myocytes and cardiac nonmuscle cells were established as described previously (15) with the exception that the DNA synthesis inhibitor bromodeoxyuridine (BrdU) was excluded from myocyte DNA experiments. For radiolabeled protein experiments, myocyte cultures were maintained in 35-mm dishes at a final cell density of 100–150 cells/mm^2 with nonmuscle cells comprising < 10% of the total cell population. All cardiac myocyte growth experiments were performed on day 1 of culture. Cardiac nonmyocyte (fibroblast) cultures were prepared from the preplates and maintained in MEM with 5% calf serum until they had proliferated to confluence (~ 4 d). Cardiac fibroblasts transformed with the SV40 large T antigen (29) were grown in MEM with 5% calf serum until confluent and used only for IL-1 proliferation experiments.

Effect of IL-1β on cardiac myocyte growth. Cells were counted at the beginning and end of each treatment period by phase-contrast microscopy to ensure constant cell numbers, as previously described (15). Cardiac myocyte growth was estimated by (*a*) continuous labeling of cell protein with ^{14}C-phenylalanine during treatment with putative growth factors and inhibitors (15), and (*b*) measuring total protein in treated and control groups by the method of Bradford (30). At least three dishes were used for each treated and control group. Chronotropy was assessed by noting beating frequency over a 2–3-min observation period on a heated (37°C) stage as described previously (27, 31). To assess the ability of IL-1 to induce the fetal/neonatal gene program, as seen with other hypertrophic agents (27, 28), skeletal α-actin and β-myosin heavy chain gene expression was assessed using a sensitive RNAse protection assay. For these experiments, ~ 2.5 μg of total RNA from control cells and cells treated with IL-1 (1 ng/ml), phenylephrine (20 μM), or calf serum (5%) was hybridized with a [^{32}P]UTP-labeled antisense probes complementary to (*a*) 195 base pairs (bp) of the last coding exon of the α-skeletal actin gene, and (*b*) a 298-bp region of the β-myosin heavy chain 3′UTR that also protects 175 bp of α-myosin heavy chain mRNA (essentially the pCMHC5 construct described in reference 32). After digestion with RNAse A/T1, protected fragments were separated on a 6% denaturing polyacrylamide gel and subjected to autoradiography as described previously (33). All signals were normalized to an internal control RNA (either 18S or β-actin) to correct for minor variations in loading and treated/control ratios derived.

To confirm that the observed effects of IL-1 were transduced through the IL-1 receptor, two mutually exclusive approaches were taken. First, we tested the ability of a 200-fold excess of IL-1RA (200 ng/ml) to block the growth response. Second, we used the 163-171–amino acid fragment of IL-1 as a potential competitive inhibitor (34). Experiments using the 163-171–fragment were carried out in the presence of increasing concentrations of peptide fragment (up to 10^6-fold excess, from 50 pg/ml to 50 μg/ml).

As noted, there has been a number of signaling pathways and second messengers described for the mitogenic actions of IL-1β in several different cell types, including the induction of prostaglandins (PG), nitric oxide (NO), and TK messenger systems. In an attempt to understand which of these mechanisms might be involved in the myocyte growth seen in our culture system, we performed a series of experiments in which specific inhibitors of these various pathways were included during IL-1β stimulation. In all experiments, inhibitor was added at least 30 min before the addition of agonist (or diluent), and the accumulation of radiolabeled protein was determined after 48 h as described above.

Effect of IL-1β on DNA synthesis and cell proliferation. To study the effects of IL-1 on myocardial DNA synthesis, both cardiac fibroblasts and myocytes were grown in 24-well plates in the absence of BrdU. Fibroblasts were grown to confluence in 5% serum (~ 5 d) before changing the medium to serum-free MEM supplemented with transferrin (10 μg/ml), insulin (10 μg/ml), and bovine serum albumin (0.1%) (TIBSA). In contrast, myocytes were taken out of serum on day 1 to prevent the increase in fibroblasts that occurs in the myocyte cultures in the absence of a DNA synthesis inhibitor (approximately twofold increase in fibroblasts after 12 h in 5% serum, and after 24 h in TIBSA). Cells were then mitogen-depleted in TIBSA for 72 h to assure quiescence and to synchronize the cells before stimulation (approximately day 7 for fibroblasts and day 4 for myocytes). Medium was then renewed, and growth factors, inhibitors (IL-1RA), or diluent was added. Fibroblast experiments were carried out both in the absence and presence of 5% calf serum to determine the effect of IL-1 on both basal and stimulated conditions of DNA synthesis, respectively. [^{3}H]Thymidine (10 μCi/ml) was added after 23 h, and incorporation into DNA was determined after 1 h as described previously (35).

Since DNA synthesis does not necessarily lead to an increase in cell number, particularly in cardiac myocytes, total cell number was also determined for both cardiac myocyte and fibroblast cultures after 24 h of IL-1 stimulation using a cell counter (Coulter Corp., Luton, Bedfords, United Kingdom). For these experiments, cells were plated into 35-mm dishes and treated in an identical fashion as described above for [^{3}H]thymidine incorporation. Cells were removed from the dishes by incubation with 1 ml of a solution of trypsin/EDTA (2 mg/ml/0.02%) for 2–5 min at 37°C. Cells were pelleted by centrifugation (500 *g*) and fixed in 25% ethanol/15 mM $MgCl_2$ followed by dilution into Isoton II (Coulter Corp.) and counting using a 140-μm aperture. Manual counts with a hemacytometer were also performed in the initial experiments to confirm the fidelity of the Coulter measurements. The disparity between fibroblast and myocyte size allowed for the assignment of myocyte versus fibroblast numbers in the myocyte cultures and, by noting an increase in cell volume in treated cultures, also confirmed IL-induced myocyte growth.

Induction of gro mRNA by IL-1β in cardiac myocytes and fibroblasts. It has been reported that, in some cell types, both the mitogenic and antimitogenic effects of IL-1β are due to the induction of the *gro* protein (36). To address this possibility in our system, we first investigated whether IL-1β treatment resulted in the induction of the *gro* peptide in cardiac cells in culture. Myocytes and fibroblasts were treated with IL-1β (1 ng/ml) for various time intervals (1–24 h). Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (37) and analyzed for integrity and concentration by size fractionation on a 1.2% denaturing agarose gel (38). 15 μg of total RNA from both myocytes and fibroblasts was separated on a denaturing 1.2% agarose gel, transferred to nitrocellulose and hybridized with a [^{32}P]UTP-labeled cDNA probe corresponding to the 3′ end of the human *gro* gene (39), washed according to the method of Church and Gilbert (40), and exposed to Kodak XOMAT film at −72°C.

Immunofluorescence. Cell cultures were prepared in the manner described above, with the cells subsequently grown on glass slides. Cells were washed with PBS and fixed with a 1:1 mixture of methanol and

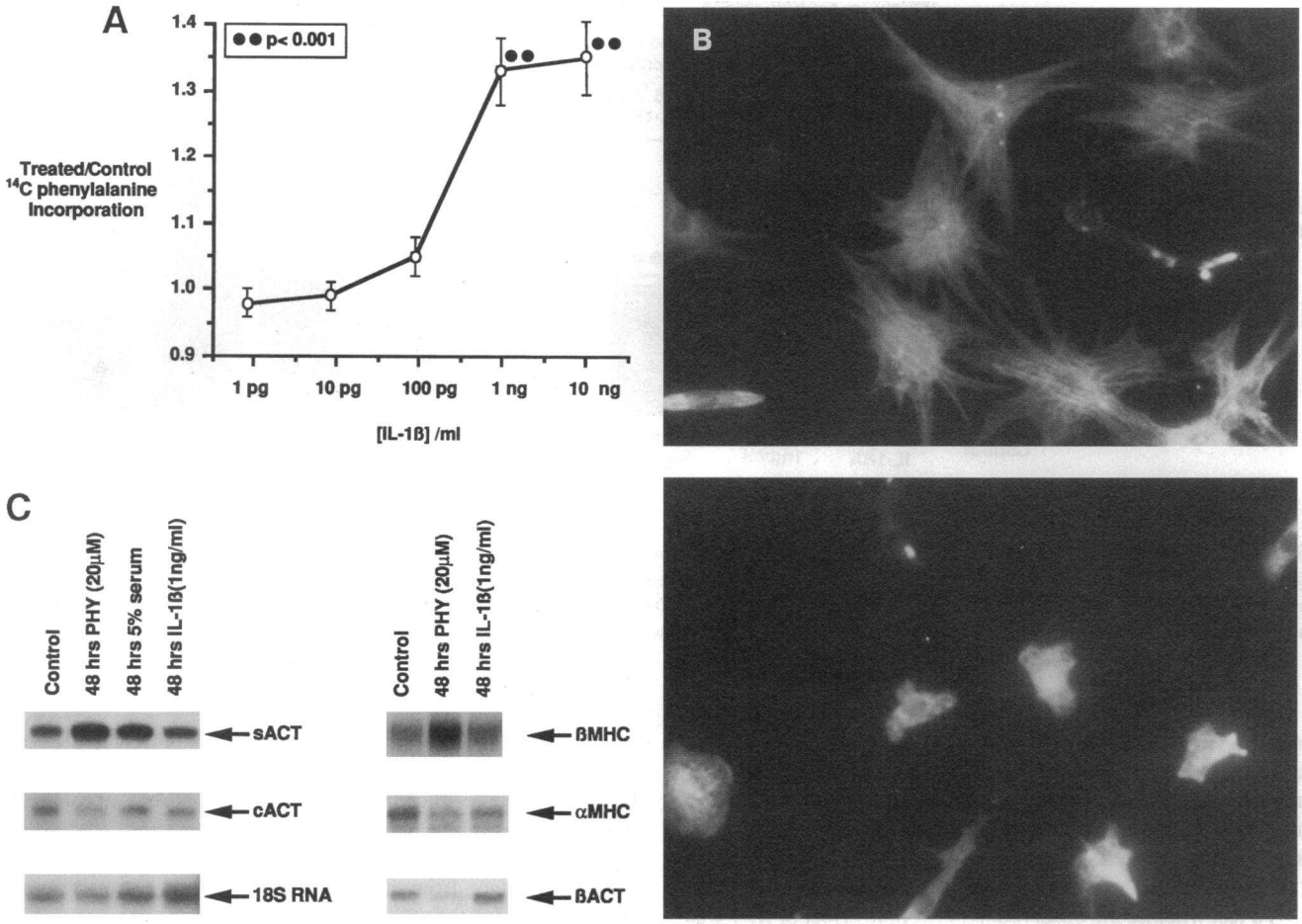

Figure 1. IL-1 stimulates cardiac myocyte growth in culture. (*A*) Cardiac myocytes were treated with increasing doses of IL-1β, and the incorporation of ^{14}C-phenylalanine into newly synthesized protein after 48 h was determined as described in Methods. Results shown represent the ratio of treated to control incorporation from five separate experiments with triplicate dishes for each dose. (*B*) Myocytes treated with IL-1β (*top*) (1 ng/ml) or diluent (PBS/BSA) (*bottom*) were fixed for immunofluorescence after 48 h. Cells were stained with antibody to striated muscle myosin heavy chain (MF-20) and photographed immediately. The staining demonstrates a noticeable increase in myocyte size and shows the cross-striations indicative of myocyte morphology. The presence of myosin bundles that reach the periphery of the cell also argues strongly that the growth induced by IL-1 is not due to a disassembly of contractile protein and an increase in nonmuscle cellular protein as has been noted for smooth muscle cells (77). (*C*) Cardiac myocytes were treated with diluent (PBS/1% BSA), IL-1 (1 ng/ml), phenylephrine (20 μM), or calf serum (5%) and 2.5 μg of total RNA hybridized with a [^{32}P]UTP-labeled antisense probe complementary to the 3′ end of the skeletal actin gene or the β myosin heavy chain gene. RNA/RNA hybrids were digested with RNAse A/T1, separated on a 6% denaturing polyacrylamide gel, and autoradiographs were obtained. The actin probe (~ 240 bp) will protect a 195-bp region of the skeletal actin mRNA (*sACT*) but is also complementary to 115 bp of the cardiac actin (*cACT*) mRNA. The myosin heavy chain probe (~ 400 bp) protects a 298-bp region of the β-myosin heavy chain mRNA and a 175-bp region of the α-myosin heavy chain RNA. Results shown are representative of at least three independent experiments.

acetone for 15 min at 4°C. After fixation, cells were incubated in 1:10 diluted normal goat serum to decrease background staining. Cell nuclei were stained with the tubulin-binding DAPI, dihydrochloride (Molecular Probes, Inc., Eugene, OR). Cardiac myocytes were delineated using a monoclonal antimyosin antibody in a 1:2 dilution (MF-20; Iowa Hybridoma Bank, Iowa City, IA). Slides were then washed with ice-cold PBS and incubated with secondary rhodamine-conjugated goat anti-mouse IgG (1:100) and viewed immediately with an inverted phase immunofluorescence microscope (Nikon).

Statistics. Results are given as mean±SEM. Mean values for two groups were compared using Student's t test, or analysis of variance for more than two groups. Treated/control ratios were tested for their deviation from unity by calculation of confidence limits (41).

Results

IL-1 induces concentration-dependent growth of cardiac myocytes. IL-1 was added to the myocyte cultures on culture day 1 in concentrations from 1 pg/ml to 10 ng/ml (57 fM–570 pM), and radiolabeled protein accumulation was determined 48 h later. Treatment with IL-1 induced a concentration-dependent increase in myocyte protein synthesis (maximum 1.38±0.04-fold over control, $n = 5$, $P < 0.001$) (Fig. 1, *A* and *B*) with an EC_{50} of ~ 250 pg/ml (14.5 pM). As an additional measure of growth, IL-1 treatment resulted in a maximal 34% (±7%) increase in total cell protein (Bradford, $n = 4$, $P \leq 0.05$) and an increase in cell volume (average 2,740 for control vs 3,280 μm^3 for IL-1β, an increase of ~ 20%). Cell proliferation did not occur in cardiac myocyte cultures during growth experiments due to addition of BrdU as noted previously (27). There were no changes in contractile activity noted with IL-1β, however, activity in control, serum-free cells was minimal (31). Using the ability of a hypertrophic agent to stimulate transcription of the skeletal α-actin and β-myosin heavy chain contractile protein genes as an indicator of the "fetal/neonatal transcriptional

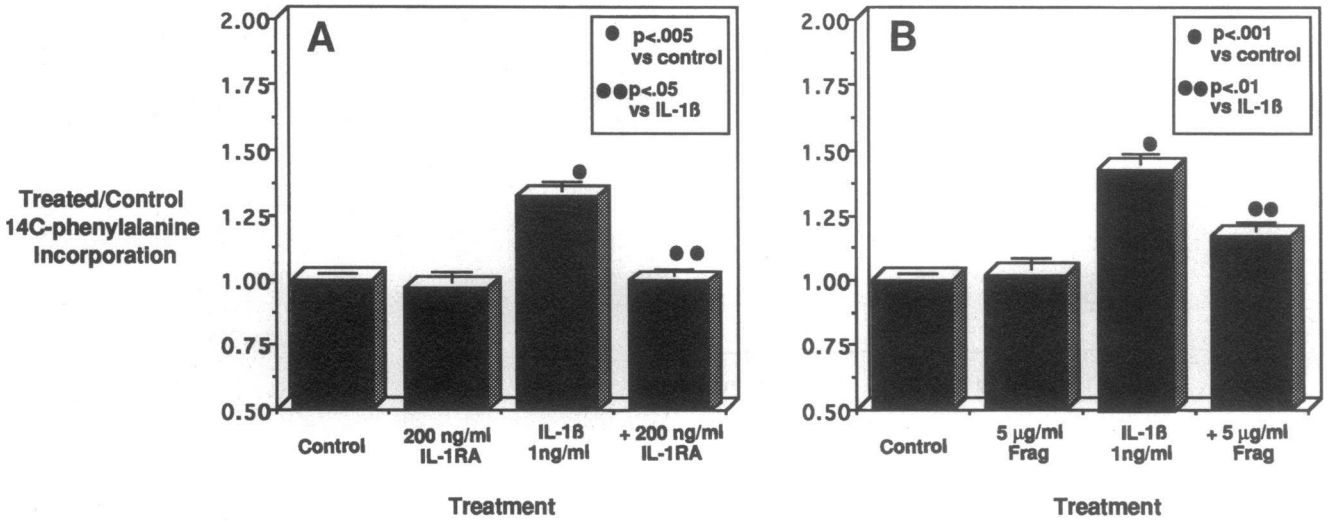

Figure 2. Inhibition of IL-1β–induced myocyte growth by IL1-RA and the 163-171–peptide fragment. After pretreatment with either IL-1RA (200 ng/ml) or the 163-171–peptide fragment (5 μg/ml), cardiac myocytes were treated with 1 ng/ml of IL-1β, and the incorporation of ^{14}C-phenylalanine into newly synthesized protein after 48 h was determined as for Fig. 1. Data shown represent $n = 5$ (IL-1RA) and $n = 4$ (163–171 fragment) independent experiments (performed in triplicate) for each condition shown.

program'' (for review see reference 42), IL-1 did not appear to act in the same way seen with other growth factors in culture in that neither skeletal α-actin nor β-myosin heavy chain mRNAs were increased (treated/control ratio of 0.95±0.06 for skeletal α-actin and 0.83±0.07 for β-myosin heavy chain, both P = NS, Fig. 1 *C*).

To test whether the observed hypertrophic effect of IL-1 was transduced through the IL-1 receptor, the naturally occurring IL-1–receptor antagonist (IL-1RA) protein was used. Control experiments indicated that, by itself, IL-1RA was neither toxic to the myocytes nor able to cause hypertrophic growth. Since it had been shown previously to require up to 500-fold excess of IL-1RA to inhibit the effects of IL-1 (43), the myocytes were treated concurrently with IL-1RA at 200 ng/ml. Under these conditions, IL-1–induced growth was prevented (Fig. 2 *A*). In addition, a synthetic 163-171–amino acid fragment of IL-1 was used as a competitive inhibitor (10^5-fold excess or 5 μg/ml). This peptide fragment of IL-1, when added in million-fold excess, has been shown to activate T cells by binding to the IL-1 receptor, but did not cause other effects associated with IL-1 stimulation (34). Control experiments indicated that the fragment was not toxic to the myocytes and did not stimulate growth. When cardiac myocytes were treated concurrently with IL-1 and the 163-171–amino acid fragment, the IL-1–induced growth effect was inhibited, although not to the extent seen with IL-1RA (Fig. 2 *B*).

Somewhat unexpectedly, we found that, in addition to an increase in protein synthesis, IL-1β was also able to reinitiate DNA synthesis in the cultured neonatal myocytes (Fig. 3 *A*). As noted above, the assessment of myocyte DNA synthesis in culture is often complicated by the observation that most of the growth factors used also have stimulatory effects on the fibroblasts that invariably contaminate these cultures. However, as noted below, in our system, IL-1β is a DNA synthesis inhibitor for fibroblasts. Since the stimulatory effect seen in the myocyte cultures could theoretically result from the paracrine action of a myocyte factor whose production was stimulated by IL-1 but whose target was, in fact, the contaminating fibroblasts, we performed conditioned medium experiments. When medium was removed from myocytes treated for 24 h with either IL-1 (IL-1 MCCM) or diluent and used to treat cardiac fibroblasts, inhibition was once again seen, indicating that the increase in myocyte [^{3}H]thymidine incorporation seen in the myocyte cultures was, in fact, from myocyte DNA synthesis. Further, inclusion of the IL-1RA protein prevented the increase in DNA synthesis seen in the myocytes (0.85±0.09 IL-1/IL-1RA versus control, $n = 3$, P = NS) and did not ''unmask'' a stimulatory action of IL-1 MCCM on the fibroblasts (data not shown). In addition, Coulter cell counts performed concurrently showed a commensurate increase in the numbers of myocytes (treated/control of 1.24±0.04, IL-1 versus diluent, $n = 3$, $P \leq 0.01$) confirming that the increase in DNA synthesis was associated with myocyte cell division. These experiments also confirmed the inhibitory effect of IL-1 on fibroblast proliferation in that numbers of myocytes, expressed as the percentage of total cells, increased over the 24-h treatment (data not shown).

IL-1–induced growth effect is mediated through a TK signaling pathway. There has been a number of signaling pathways and second messengers described for the mitogenic actions of IL-1β in several different cell types. In several cases, both IL-1's stimulation and inhibition of cell proliferation have been attributed to the autocrine production/release of PDGF and upregulation of the PDGF α receptor or the release of growth-inhibitory prostanoids, respectively (44–46). Other mechanisms include the direct induction of prostaglandins (47, 48), NO (5, 9, 49), PKC (50, 51), and TK-phosphorylated proteins (25, 26). In the cardiovascular system, previous work has suggested that it is the NO generated in response to IL-1 stimulation that is responsible for both the negative inotropic as well as chronotropic response of the heart (7–9, 11, 52). To address these possibilities, growth experiments were repeated in the presence and absence of the NO synthase inhibitor NΩ-nitro-L-arginine methyl ester, the PKC inhibitor staurosporine, the cyclooxygenase inhibitor indomethacin, and the TK inhibitor genistein (Table I). Indomethacin, a cyclooxygenase inhibitor, failed to block the growth effect of 1 ng/ml of IL-1β even at 100 μM, a concen-

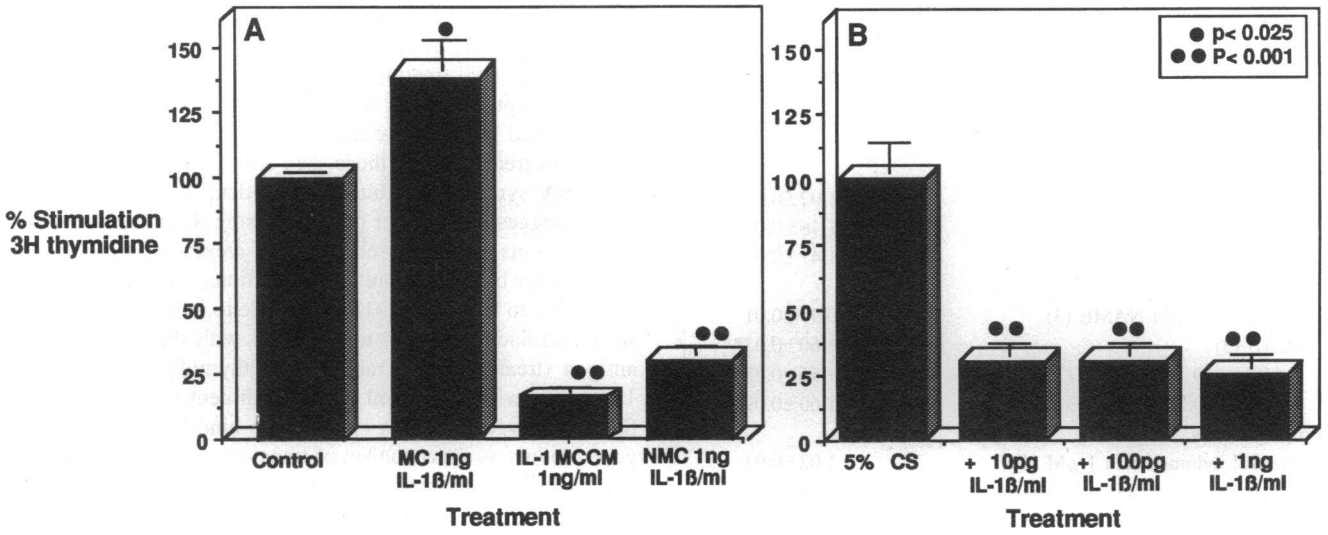

Figure 3. IL-1β differentially regulates myocardial DNA synthesis in culture. Cardiac fibroblasts and myocytes were grown in 24-well dishes in the absence of BrdU. Cells were mitogen depleted in TIBSA for 72 h to assure quiescence and to synchronize the cells before stimulation (approximately day 7 for fibroblasts and day 4 for myocytes). [^{3}H]Thymidine (10 μCi/ml) was added after 23 h, and incorporation into DNA was determined after 1 h. (*A*) Effect of IL-1 on basal thymidine incorporation. Values shown are relative to the [^{3}H]thymidine incorporation at 23 h of cells treated with diluent alone (100%). Results are from $n = 5$ ($n = 3$ for IL-1MCCM) separate experiments (performed in triplicate) per condition. For the IL-1MCCM experiments, the 23-h myocyte medium containing 1 ng/ml of IL-1β was removed from the myocytes and added directly to confluent, mitogen-depleted cultures of fibroblasts. Control for the MCCM experiments was both 23 h, diluent-treated MCCM and freshly added diluent (no inhibition noted with either). (*B*) Effect of IL-1 on mitogen-stimulated thymidine incorporation. Values shown are relative to the [^{3}H]thymidine incorporation at 23 h of cells treated with 5% calf serum (100%). Note that, by itself, serum induces a nearly fourfold increase over control cells at 23 h. Results shown are from $n = 5$ separate experiments (performed in triplicate) per condition.

tration 10-fold above that found previously to inhibit prostaglandin production in the cardiac myocytes (Simpson, P. C., unpublished data). IL-1 has also been reported to cause upregulation of inducible nitric oxide synthase (iNOS) enzyme system. We used two approaches to identify the possible role of the NO pathway on the induction of myocyte hypertrophy by IL-1. $N\Omega$-nitro-L-arginine methyl ester has been shown to block the action of iNOS in rat neonatal cardiac myocytes (7, 52), but even at the maximal dose reported (100 μM), no inhibition of the growth-response was noted. Similarly, using sodium nitroprusside (SNP, 1 μM–1 mM) which induces NO expression and cGMP upregulation, no increase in radiolabeled protein was seen (0.84±0.14-fold over control cells, $n = 3$, p = NS for the 1 mM dose). To address the role of the PKC pathway the action of IL-1, the PKC inhibitor staurosporine was used at 5 nM, a concentration shown previously to block the PKC-dependent cardiac myocyte growth effect of both PMA and phenylephrine (53). At this concentration, staurosporine was not able to block IL-1–induced growth.

In contrast, use of the TK inhibitor genistein at a concentration near the IC_{50} for EGF receptor phosphorylation and IL-1–stimulated PGE_2 in mesangial cells (48, 54) resulted in inhibition of the IL-1–induced growth effect. This concentration resulted in a similar inhibition of the myocyte growth effect of platelet-derived growth factor (PDGF), a peptide known to act via a TK-linked cell surface receptor. PDGF induces a similar degree of hypertrophy in cardiac myocytes (~ 1.5-fold increase in myocyte protein synthesis) as IL-1. In contrast, the growth effect of 1% calf serum (a concentration with equivalent myocyte growth induction to IL-1β) was unaffected by the inclusion of genistein (Fig. 4).

IL-6 and gIFN do not induce cardiac myocyte hypertrophy in culture. To explore the hypothesis that the IL-1–induced hypertrophic effect may be unique among cytokines, myocytes were also treated with increasing doses of both IL-6 and γIFN, two other factors known to be actively involved in the immune response. In contrast to the findings seen with IL-1, neither IL-6 nor γIFN was capable of increasing myocyte protein synthesis (^{14}C-phenylalanine) over 48 h of treatment (1.03±0.04 for 100 ng/ml IL-6 vs control and 1.01±0.03 vs control for 100 ng/ml γIFN), despite the known expression of receptors for both on cardiac myocytes.

IL-1 inhibits the proliferation of rat cardiac fibroblasts. Previous work from this and other laboratories has shown that the predominant nonmuscle cell type of the heart, the cardiac fibroblast, is the source of a number of important growth factors that may play a role in myocardial growth and development. Because these cells outnumber the myocytes in the normal heart and are largely responsible for the extracellular matrix of the heart and the connective tissue response to injury, we were also interested in the response of these cells to IL-1. Since the cardiac fibroblasts respond to stimuli largely through proliferation, we investigated the [^{3}H]thymidine uptake of the fibroblasts after IL-1 treatment. Surprisingly, we found that IL-1 actually inhibits its basal levels of fibroblast proliferation (Fig. 3 *A*). Similar to that seen with myocyte DNA synthesis, inclusion of IL-1RA during the treatment period prevented the inhibition of [^{3}H]-thymidine incorporation confirming the direct effect of IL-1 in this process (data not shown). Because IL-1 inhibited basal fibroblast proliferation, we next asked whether IL-1 might inhibit growth factor stimulation of fibroblast DNA synthesis. For these experiments, cultures were stimulated for 24 h with 5% calf serum in both the presence and absence of increasing doses of IL-1 (10 pg–1 ng). As indicated in Fig. 3 *B*, IL-1 inhibited the serum-stimulated increase in DNA synthesis by > 50% ($n = 5$). Since both of these findings could result from an IL-

Table I. Second Messenger Pathways in IL-1β–stimulated Cardiac Myocyte Hypertrophy

Second messenger pathway treatment (*n*)	Treated/Control radiolabeled protein±SEM
PKC	
Control staurosporine 5 nM (5)	1.02±0.04
IL-1β (5)	1.46±0.07*
IL-1β + staurosporine (5)	1.47±0.07
NO	
Control 100 μM NAME (3)	0.97±0.01
IL-1β (3)	1.60±0.03*
IL-1β + 10 μM NAME (3)	1.56±0.05
IL-1β + 100 μM NAME (3)	1.60±0.09
Prostaglandins	
Control indomethacin 1 μM (4)	1.02±0.03
Control indomethacin 10 μM (4)	1.05±0.02
IL-1β (4)	1.52±0.09‡
IL-1β + 1 μM indomethacin (4)	1.38±0.10
IL-1β + 10 μM indomethacin (4)	1.38±0.03
Tyrosine kinase	
Control 10 μM genistein (7)	1.02±0.04
IL-1β (7)	1.51±0.04*
IL-1β + 10 μM genistein (7)	1.26±0.03§

Myocytes were treated with IL-1β (1 ng/ml) or diluent (PBS/0.1% BSA or DMSO) for 48 h in the presence or absence of the indicated inhibitors. Incorporation of ^{14}C-phenylalanine into newly synthesized protein was determined as described in Methods. Control cells were treated with inhibitor to assure that the results observed in the presence of IL-1β were not due to the toxicity of diluent (i.e., DMSO). No differences were seen in control cells treated with diluent alone versus inhibitor alone. ‡ $P < 0.025$ and * $P < 0.005$ for IL-1β cells versus control cells, § $P < 0.001$ for IL-1β treated versus IL-1β/genistein cells. All other ratios did not reach statistical significance when compared with IL-1β treatment alone.

1 induction of programmed cell death (apoptosis) in cardiac fibroblasts, we examined the effect of IL-1 on total cell numbers using an identical treatment protocol to that used for the [^{3}H]-thymidine uptake. Under these conditions, cell numbers in the IL-1–treated groups were unchanged compared with those on the day of treatment and those seen after 24 h of treatment with the DNA synthesis inhibitor BrdU (data not shown). These results suggest that, rather than increasing fibroblast cell death, IL-1 prevents cardiac fibroblasts from reentering the cell cycle either under basal or stimulated conditions. In this regard, it is interesting to note that IL-1β is not able to inhibit DNA synthesis in cardiac fibroblasts transformed with the SV40 large T antigen (treated/control ratio of [^{3}H]thymidine incorporation 1.10±0.14 with 100 pg/ml IL-1β vs diluent, $n = 5$, P = NS). Since the large T antigen is known to bind the retinoblastoma gene product as a mechanism of transformation, this result suggested the possible involvement of the retinoblastoma protein in the IL-1–induced inhibition in our primary cells as has been reported in other systems with TGFβ (55, 56).

Unfortunately, we were not able to address with certainty the effect of TK inhibition on the fibroblast response to IL-1 due to the toxic effect of the diluent, DMSO, on control thymidine incorporation (~ 90% inhibition of [^{3}H]thymidine incorporation compared with non–DMSO-treated cells). Despite this basal inhibition, however, if compared with genistein-treated control cells, IL-1 was still able to inhibit fibroblast [^{3}H]thymidine by ~ 80%, ([^{3}H]thymidine incorporation 0.14±0.03 in IL-1–treated cells vs control compared with 0.20±0.06 in IL-1 plus genistein–treated cells vs genistein-treated control cells, $n = 3$, P = NS compared with IL-1 alone) suggesting that the TK pathway was not involved in the IL-1 inhibition of fibroblast DNA synthesis. The prostaglandin inhibitor indomethacin was also unable to block the effect of IL-1 on the fibroblasts, suggesting that growth-inhibitory prostaglandins were not the mechanism of action either.

It has been reported previously that both the mitogenic and antimitogenic effects of IL-1 in diverse cell lines are associated with the induction of the *gro*/melanoma growth-stimulatory ac-

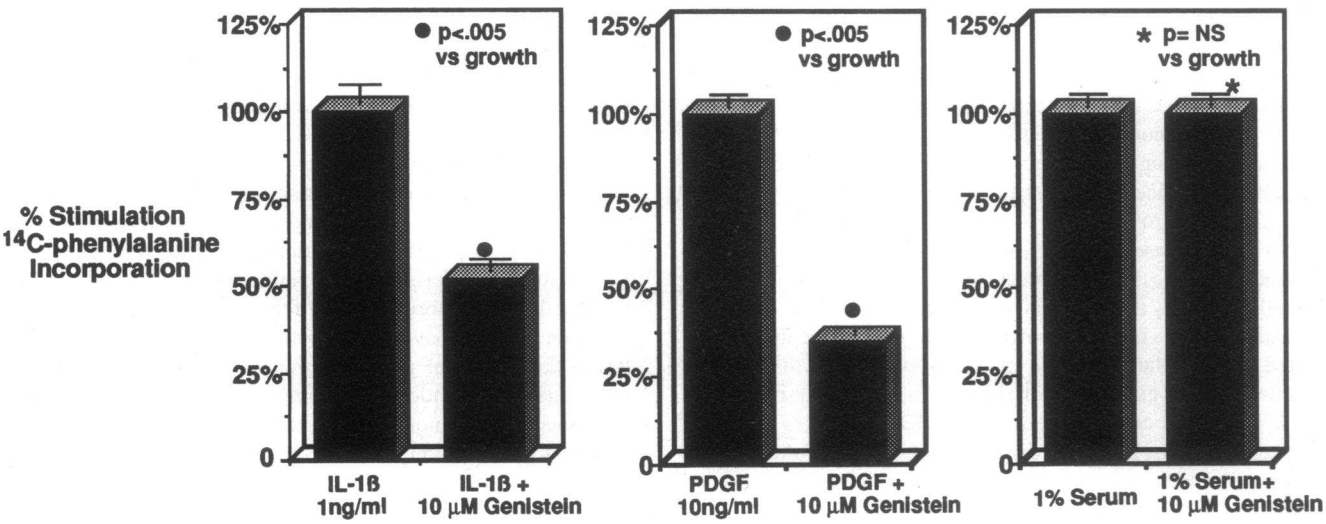

Figure 4. Genistein inhibits IL-1β and PDGF, but not serum-induced cardiac myocyte growth. Cardiac myocytes were treated with IL-1β (1 ng/ml), PDGF (10 ng/ml), or 1% calf serum for 48 h in the presence or absence of the TK inhibitor genistein (10 μM). Incorporation of ^{14}C-phenylalanine into newly synthesized protein was determined and compared with cells treated with diluent. Data shown represent the average of at least three different experiments with triplicate dishes for each condition.

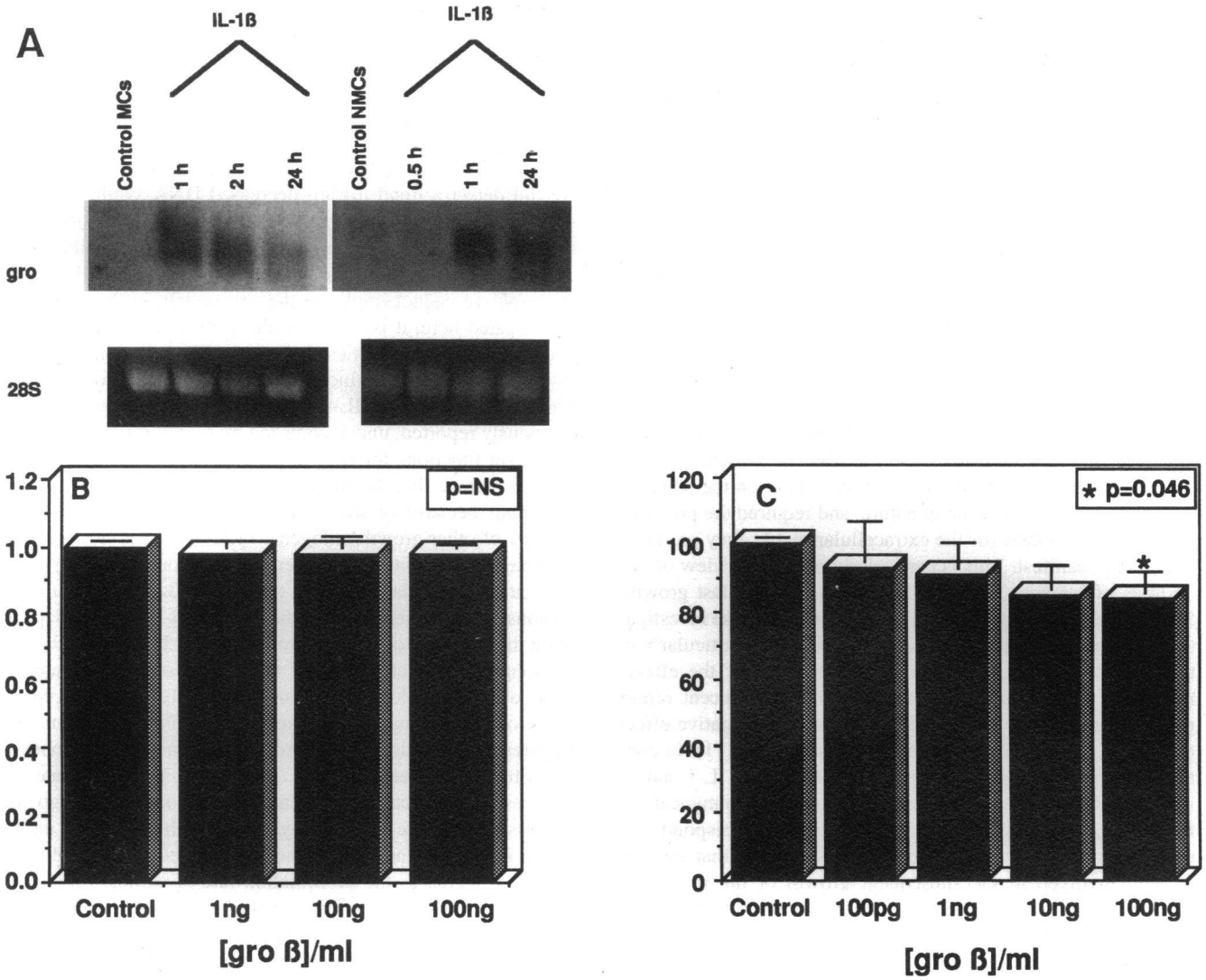

Figure 5. gro expression and effect in cardiac myocytes and fibroblasts in culture. (*A*) *gro* mRNA expression. Cardiac myocytes and fibroblasts were treated with IL-1β (1 ng/ml) for the indicated times and 15 μg of total RNA from each cell type separated by a denaturing 1.2% agarose gel. RNA was transferred to nylon and hybridized with a cDNA complementary to the human *gro* gene. After washing as described, membranes were exposed to Kodak XOMAT film at −72°C for 24–36 h. The signal shown is of the correct size(s) for the *gro* gene family (900/1,100 bp). (*B*) *gro* growth effects. Cardiac myocytes were treated with increasing doses of rat *gro* β, and the incorporation of ^{14}C-phenylalanine into newly synthesized protein after 48 h was determined. Results shown represent the ratio of treated to control incorporation from six separate experiments with triplicate dishes for each dose. (*C*) Cardiac fibroblast [^{3}H]thymidine incorporation in response to rat *gro* β was performed as described for the IL-1β experiments. Results shown represent the ratio of treated to control incorporation from five separate experiments (n = 4 for 100 ng/ml) with triplicate dishes for each dose.

tivity (MGSA) protein (36), a member of the chemokine family. Because of this precedence, and our findings of a differential cell response to IL-1 by cardiac myocytes and fibroblasts, we investigated both the expression of *gro* mRNA in the myocytes and fibroblasts and the effect of purified *gro* protein on both cell types. As indicated in Fig. 5 *A*, IL-1 induced a transient increase in *gro* mRNA in both cardiac myocytes and fibroblasts with message easily being detected at the 1 h time point. However as shown in Fig. 5 *B*, addition of purified *gro* protein was not able to reproduce the hypertrophic effect on the myocytes even when added at 100 ng/ml. There was, however, a trend toward a mild suppressive effect on the cardiac fibroblasts, although this was only significant (P = 0.046) when added at 100 ng/ml.

Discussion

The major finding of this work is that IL-1 exhibits differential growth regulation of the two main cells types that make up the heart, namely cardiac myocytes and cardiac fibroblasts. Specifically, IL-1 induces hypertrophy and DNA synthesis of cardiac myocytes, while repressing fibroblast proliferation. These findings are particularly noteworthy given that cardiac fibroblasts are capable of producing IL-1 in response to both endotoxin (LPS) and hypoxia/reperfusion in culture (17).

Although IL-1 is primarily known as a mediator of inflammation, it also plays many other diverse physiologic roles including both stimulation and inhibition of proliferation of both primary and transformed cells (44, 45, 57–59), including inter-

stitial (i.e., fibroblasts) and parenchymal cells of a number of organs. In the heart, IL-1 expression has traditionally been reported in situations involving immunologic myocardial injury such as occurs during transplant rejection, reperfusion injury, and congestive heart failure (2–4, 60–63). Based on these reports, the effects of IL-1 have been presumed to be negative in nature, a conclusion seemingly supported by the observations of others using both high density myocyte cultures (7, 8, 64) and experimental models in which cytokine infusion studies of both in situ and explanted hearts and papillary muscles were performed (1, 6). It is important to note that in one of the former reports, the negative chronotropic effect of IL-1 in culture was due to the production of NO, although TNFα, an equally potent inducer of NO synthase did not have a similar detrimental effect on intrinsic myocyte beating rate suggesting that "NO is only one of several factors important in the regulation of myocyte contractility" (7). Viewed in combination with our findings, it is noteworthy that both Roberts et al. (8) and Hosenpud et al. (64) suggested that the negative effects of IL-1 on the neonatal myocytes may be paracrine in nature, and required the presence of cardiac fibroblasts (or the extracellular matrix they produce) to become manifest. This is particularly relevant in view of our findings of a potent negative IL-1 effect on fibroblast growth. Since the high density myocyte cultures used by these investigators do contain increased numbers of fibroblasts (particularly in the absence of BrdU), it is possible that some of the effects may have been fibroblast in origin, although a recent report using adult rat myocytes has suggested a direct negative effect of IL-1 on adrenergic-stimulated myocyte beating (11). In contrast, our results suggest that cardiac expression of IL-1 and its subsequent effects may not always be deleterious to myocardial function. Our findings suggest that the heart may respond to a pathologic stress with the production of factor(s) that may be directly involved in the subsequent growth of the remaining myocytes while at the same time limiting the fibroblast proliferation that could lead to an overly exuberant scarring response. Multiple other growth/mitogenic factors, such as TGFβ, also appear in myocardium in response to injury, and the expression of such factors is likely to be involved in the healing/repair that follows. Specifically, the expression of both FGF and VEGF has been postulated to be involved in post-injury angiogenesis, an effect that also extends to some interleukins in other circumstances (65–68). Such post-infarct growth factor/cytokine expression may reflect the complex way that the heart can respond to injury in a positive, more adaptive, way. The present work suggests that there may be a role for IL-1β in this process as well.

In an attempt to understand the mechanism through which IL-1 exerts its hypertrophic effects, we investigated the role of a number of the previously described IL-1 signaling pathways in our culture system. Although the IL-1 protein is known to act through a number of different mechanisms in other cell types, the induction of cardiac myocyte hypertrophy appears to involve a TK pathway. Unlike that seen with rat aortic smooth muscle cells (49) however, it does not appear that the IL-1–induced myocyte TK activity involves NO as the final effector of the hypertrophic growth, a supposition that is supported by the lack of growth seen in response to the NO donor, sodium nitroprusside. However, IL-1 is capable of inducing both myocyte iNOS and the subsequent production of NO in cultures of cardiac myocytes and fibroblasts (7, 8, 11, 52), although the exact effect of this production is not entirely clear at this time. Unfortunately, we were not able to address with certainty the role of TK signaling in the antiproliferative effect of IL-1 on the cardiac fibroblasts because of the negative effect of DMSO alone on [^{3}H]thymidine incorporation. It is clear, however, that the decrease in DNA synthesis was not due to the autocrine action of induced prostaglandin production as has been reported in other cell types (for review see reference 69). The role of the *gro* protein in fibroblast proliferation is less clear. Although we did detect a trend toward decreased DNA synthesis in response to recombinant rat *gro*, this only occurred at a concentration 1,000–10,000-fold that of IL-1 required for inhibition, a concentration unlikely to be present physiologically. However, although we cannot implicate the *gro* protein in the processes investigated here, it is a known chemokine with potent leukocyte chemotactic properties (70). As such, our finding that both myocardial cell types increase their expression of the *gro* mRNA in response to IL-1, an observation that has not been previously reported, underscores the myriad of other potentially important functions for IL-1 in the myocardium.

We believe that the findings reported here are particularly important because of the observations that, in addition to a number of other growth/mitogenic factors, IL-1 is expressed in myocardial cells both after ischemia/infarction and during acute myocarditis/transplant rejection (2–4, 60–63, 71). The ultimate response of the heart to such pathologic states is likely to depend upon the interplay of many factors that may have both additive and opposing effects on all of these processes. For example, areas of myocyte loss that occur after infarct are replaced by areas of fibroblasts and collagen deposition, a situation that requires the activation of both proliferative and secretory phenotypes for these interstitial cells. In addition, to compensate for the loss of contractile cells, remaining myocytes undergo the process of myocyte hypertrophy (either with or without an increase in myocyte numbers). The work presented here indicates that IL-1 decreases the proliferation rate of cardiac fibroblasts in culture, including growth factor–mediated proliferation. However, although one might expect this to result in an increase in extracellular matrix gene expression (a secretory phenotype for fibroblasts), no increase in mRNA levels of the collagen I and III isoforms was seen in response to IL-1 in these cells (Long, C. S., and W. Hartogensis, unpublished observations). The myocyte effects of IL-1, however, appear to be more adaptive in nature, resulting in myocyte growth which, at least in terms of contractile protein gene expression (actin and myosin heavy chain), indicates a mechanism of induced growth which is dissimilar to that seen with most of the other hypertrophic agents. The functional consequences of such diversity in induced gene expression during hypertrophy are unknown. However, they may indicate a fundamental difference in the ability of cardiac myocytes to adapt to varied forms of stress in vivo.

Although we did not detect any changes in myocyte beating with IL-1 treatment in our low density cultures, it is interesting to speculate that the decrease in contractility/chronotropy seen in response to cytokines such as IL-1 in vitro may be responsible, at least in part, for the stunning and hibernation processes that occur with myocardial injury acutely and chronically, respectively. Such a decrease in contractility would be advantageous in the sense of that it would result in a decrease in energy requirements at a time of decreased substrate supply. This concept is consistent with the findings of IL-1 suppression of both intrinsic and stimulated contractility seen with both neonatal and adult cells in vitro (8, 11, 72). In this way, our findings do not require the discarding of previous experimental observations on cytokine actions, but rather a reinterpretation of findings in

response to an even larger body of literature describing the phenomenon of post-injury myocardial adaptation and growth factor/cytokine gene expression. Although the idea of cytokines acting in a therapeutic or even physiologic manner is certainly a novel one, a role for IL-6 and NO in the stunning process has been suggested by others (73, 74). In addition, in vitro data support the notion that cytokines such as IL-1 and TNFα may have beneficial effects during times of ischemic stress (75, 76). The investigators attribute this protective effect to an increase in the ability of the myocardium to deal with oxidative metabolites/free radicals via the production of increased scavenging enzymes such as MnSOD. We believe that this apparent cardioprotective effect, along with the observations reported here, indicate that IL-1 has multiple effects on the myocardium, many of which may be physiologic.

It is important to note that the present report deals specifically with the effects of IL-1 on neonatal myocardial cells. As such, it is possible that our findings cannot be extrapolated to the adult heart. However, the findings that IL-1 expression is increased in adult hearts in response to injury (2–4, 60–63) and that adult cells can respond to exogenous IL-1 (11) suggest that the two systems may act similarly. Further, these observations provide the substrate for future studies aimed at investigating the role of IL-1 in the process of myocardial growth/proliferation in mature hearts.

In summary, IL-1 appears capable of inducing what may be termed as an adaptive form of myocardial hypertrophy, i.e., the induction of myocyte hypertrophy (possibly with an increase in myocyte number) while inhibiting cardiac fibroblast proliferation and possibly the extracellular matrix/scar that these cells produce. Perhaps more importantly, this work underscores the importance of investigating the complex way that the cells that make up the mature heart are both the source and the target of many of the previously described peptide growth factors and cytokines.

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