

# Proinflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism

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**Panas, Donna, Fadi H. Khadour, Csaba Szabó, and Richard Schulz.** Proinflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H1016–H1023, 1998.— Proinflammatory cytokines (interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$ ; Cytomix) depress myocardial contractile work partially by stimulating expression of inducible nitric oxide (NO) synthase (iNOS). Because NO and peroxynitrite inhibit myocardial O<sub>2</sub> consumption (MV̇O<sub>2</sub>), we examined whether this mechanism contributes to reduced cardiac work. In control isolated working rat hearts, cardiac work was stable for 60 min, followed by a decline from 60 to 120 min, without change in MV̇O<sub>2</sub>. Cardiac efficiency (work/MV̇O<sub>2</sub>) was therefore reduced from 60 to 120 min. Cytomix shortened the onset (within 20–40 min) and enhanced the depression in cardiac work and efficiency and inhibited MV̇O<sub>2</sub> after 80 min. Mercaptoethylguanidine (MEG), an iNOS inhibitor and peroxynitrite scavenger, or the glucocorticoid dexamethasone (Dex) abolished the effects of Cytomix. iNOS expression was increased 10-fold by Cytomix and abolished by Dex but not MEG. That cytokine-induced depression in cardiac work precedes the reduction in MV̇O<sub>2</sub> suggests, at least in the early response, that NO and/or peroxynitrite may not impair heart function by inhibiting mitochondrial respiration but reduce the heart's ability to utilize ATP for contractile work.

inducible nitric oxide synthase; peroxynitrite; mercaptoethylguanidine; dexamethasone; isolated heart

THE FREE RADICAL GAS nitric oxide (NO) is synthesized in the normal heart by cardiac myocytes (2, 33), vascular endothelial cells (25), endocardial cells (35), and some cardiac neurons (46). The constitutive isoform of NO synthase (NOS) present in endothelial cells and cardiac myocytes is endothelial NOS (eNOS) (2), whereas that in cardiac neurons is the neuronal isoform (46). The physiological production of NO in the heart serves as a signaling pathway to regulate coronary vascular tone (18), prevents platelet activation (31), and regulates cardiac muscle function, with both negative inotropic (6, 26) and chronotropic actions (15). In the heart, NO acts as an endogenous inhibitor that counteracts the increase in contractile function through  $\beta$ -adrenergic stimulation (2, 16). Many of these physiological actions of NO are mediated, at least in part, by stimulation of the soluble guanylate cyclase.

A depression of cardiac contractile function during inflammatory conditions, such as septic shock, is mediated by proinflammatory cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (8, 23, 38). Endogenous release of these proinflammatory cytokines in response to bacterial lipopolysaccharide is responsible for the expression

of the Ca<sup>2+</sup>-independent, inducible isoform of NO synthase (iNOS) in a variety of cells and tissues in vivo (22). Enhanced formation of NO by iNOS has been implicated in the cardiovascular alterations associated with immunostimulation and septic shock (for review, see Ref. 40). We have previously demonstrated the de novo expression of iNOS activity in cardiac myocytes treated with cytokines in vitro or in ventricular wall homogenates from rats treated with bacterial lipopolysaccharide in vivo (33), with iNOS mRNA expression occurring within as little as 30 min in the latter case (4). The expression of iNOS activity either in vitro or in vivo was prevented by the glucocorticoid dexamethasone (Dex) (33). iNOS expression in the heart contributes to the depression of contractile function in isolated cardiac myocytes (3, 5), papillary muscles (11), and the intact heart (34) as well as to the cytolysis of cardiac myocytes (27). Each of these consequences of the expression of iNOS activity can be attenuated with isoform nonselective inhibitors of NOS, i.e., N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME).

The exact mechanism by which enhanced NO production in the heart leads to mechanical dysfunction is unknown. Because NO and its product with superoxide, peroxynitrite, are known to inhibit O<sub>2</sub> consumption in cardiac muscle slices (50), we tested the hypothesis that depression of mechanical function was secondary to depression in myocardial O<sub>2</sub> consumption (MV̇O<sub>2</sub>).

Development of NOS inhibitors selective for iNOS should allow a specific targeting and reduction of iNOS activity without affecting the essential actions of the constitutive NOS isoforms (eNOS and neuronal NOS). Mercaptoethylguanidine (MEG) is a novel NOS inhibitor that has been shown to have selectivity toward iNOS compared with eNOS and prevented hypotension in rat endotoxin shock (39). MEG also has additional actions as a peroxynitrite scavenger (41) and is therefore an effective tool in preventing cytotoxic effects related to NO overproduction and peroxynitrite generation. In this study, we tested whether MEG could prevent cardiac depression in isolated working rat hearts exposed to the combination of IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  (Cytomix) and compared its effects with those of the glucocorticoid Dex.

## METHODS

This investigation conforms with *The Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care (revised 1993).

**Heart perfusions.** Male Sprague-Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbi-

tal sodium (60 mg/kg). The hearts were rapidly excised, cannulated via the aorta, and initially perfused in a retrograde manner (Langendorff method) with Krebs-Henseleit buffer at 37°C, continuously gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. During this initial perfusion, the hearts were trimmed of excess tissue, and both the pulmonary artery and the opening to the left atrium were cannulated. After a 10-min equilibrium period, the perfusion of the heart was switched to the working heart mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow and aortic outflow lines. The working heart perfusate (recirculating Krebs-Henseleit solution containing 11 mM glucose, 5 mM pyruvate, 100 μU/ml insulin, 1.75 mM Ca<sup>2+</sup>, 0.5 mM EDTA, and 0.2% BSA) was delivered from the oxygenator (supplied with 95% O<sub>2</sub>-5% CO<sub>2</sub>) into the left atrium at a hydrostatic preload equivalent to 9.5 mmHg. Hearts were paced at 300 beats/min throughout the experiment with a Grass SD9 stimulator (regular stimuli, duration 0.6 ms, delay 0.4 ms) with leads placed on the aortic and left atrial cannulas. The perfusate (pH 7.4, 37°C) was ejected from the hearts into a compliance chamber (containing 1 ml of air) and into the aortic outflow line. Heart rate and peak systolic pressure were measured with a TSD104 Grass pressure transducer in the aortic outflow line and recorded in real time by using the AcqKnowledge III data acquisition system (Biopac Systems, Goleta, CA). The hydrostatic afterload pressure was set at a column height equivalent to 70 mmHg. Cardiac output and aortic flow were measured by using ultrasonic flow probes (Transonic) in the preload and afterload lines, respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. MVO<sub>2</sub> was calculated according to the Fick principle on the basis of coronary flow rates and the difference between left atrial and pulmonary artery O<sub>2</sub> content measured by using O<sub>2</sub> microelectrodes (Yellow Springs Instrument, Yellow Springs, OH) and expressed as a rate per gram of dry heart weight. Cardiac efficiency was determined as the ratio of cardiac work to O<sub>2</sub> consumption rate uncorrected for heart weight.

**Experimental protocol.** After 20 min of equilibration in the working mode, cardiac output, aortic pressure, and coronary flow were measured. Cardiac work, the product of cardiac output (ml/min) × peak systolic pressure (mmHg), was used as an index of mechanical function. Coronary conductance was determined as the ratio of coronary flow over mean aortic pressure (diastolic pressure + 1/3 pulse pressure). Cytomix, a combination of IL-1β (5 ng/ml), TNF-α (20 ng/ml), and IFN-γ (9 ng/ml), with or without MEG (0.3–30 μM), was added to some hearts (referred to as *t* = 0 h), and the hearts were perfused for 2 h. Other hearts were treated with Dex (3 μM) at the beginning of perfusion in the working mode. An additional group of hearts was perfused without addition of cytokines in the presence of Dex (3 μM) or MEG (1 or 30 μM). At the end of the perfusion protocol (*t* = 2 h), heart ventricles were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid N<sub>2</sub> and then stored at -80°C for later processing.

The frozen ventricular tissue was then weighed and powdered with a pestle and mortar cooled to the temperature of liquid N<sub>2</sub>. A portion of the powdered tissue was used to determine the dry-to-wet weight ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 h at 100°C, and weighed. The dried atrial weight, frozen ventricular weight, and ventricular dry-to-wet weight ratio were then used to determine the total dry weight of the heart.

**Preparation of cytosolic fraction from frozen ventricle.** The frozen ventricular tissue was powdered with a pestle and mortar cooled to the temperature of liquid N<sub>2</sub>. A portion of this

was weighed out and placed in 4 vol (wt/vol) of ice-cold homogenization buffer (composition given in Ref. 33) and homogenized by using an Ultra-Turrex disperser with three strokes of 6-s duration each. The homogenate was centrifuged (1,000 *g* for 5 min) at 4°C, and the supernatant fraction was kept. Protein content was measured by the bicinchoninic acid method, with BSA as a standard. The supernatant was diluted with 100 of μl protein sample buffer (30% vol/vol glycerol, 6% wt/vol SDS, 0.13 M Tris, 0.1 mg/ml bromphenol blue, and 3% vol/vol 2-mercaptoethanol, pH 6.8) and with homogenization buffer to give a final protein concentration of 3 μg/μl in a total volume of 300 μl. The samples were boiled for 5 min and then stored at -20°C.

**Western blot analysis.** SDS-PAGE was performed with 90 μg of protein loaded per lane on homogeneous gels (9% acrylamide) by using a Mini-Protean II electrophoresis cell (Bio-Rad) at a constant voltage of 120 V. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45-μm pore size, Bio-Rad) for 2 h at 100 V by using a transfer buffer (25 mM Tris, 192 mM glycine, and 25% vol/vol methanol). The transfer cell was kept on ice. The membrane was then blocked with 10% skim milk powder (wt/vol) in PBS for 4 h at room temperature. The blot was then incubated for 2 h with rabbit polyclonal antiserum to rat and mouse iNOS, diluted 1:1,000 in blocking buffer (1% wt/vol skim milk powder in PBS). The blots were washed twice for 5 min in PBS containing 0.05% vol/vol Tween 20 and 5 min in PBS before incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted 1:2,000 with blocking buffer. After that, the blots were washed three times for 5 min each in PBS containing 0.05% vol/vol Tween 20 and three times for 5 min in PBS. All of the membrane processing was done at room temperature with constant agitation. The protein bands detected by the antibodies were visualized using the reagents from an enhanced chemiluminescence detection kit (Amersham) and were autoradiographed using scientific imaging film (Kodak). As a positive control for detection of iNOS, a cytosolic liver extract was prepared as above from rats treated for 6 h with endotoxin (*Salmonella typhosa* lipopolysaccharide, DIFCO, 4 mg/kg ip; Ref. 22). Band densities were analyzed by National Institute of Health image software (Wayne Rosband, National Institute of Mental Health, Bethesda, MD).

**Materials.** Recombinant human IL-1β and TNF-α (Upjohn, Kalamazoo, MI), and rabbit polyclonal antiserum to mouse and rat macrophage iNOS (Dr. Richard Mumford, Merck Research Laboratories, Rahway, NJ) were kind gifts from the sources indicated. Rat IFN-γ was purchased from GIBCO BRL (Burlington, ON, Canada). All other reagents were obtained from Fisher Scientific or Sigma. Solutions of MEG were prepared fresh daily as previously described (39).

**Statistics.** Results are expressed as means ± SE for *n* experiments. As appropriate, Student's unpaired *t*-test, Welch's test, or one-way ANOVA, followed by Tukey's test to compare individual means, was used for statistical comparisons. *P* < 0.05 was used as the criterion for statistical significance.

## RESULTS

**Time course of cytokine-induced changes in cardiac work, O<sub>2</sub> consumption, and efficiency.** Figure 1A shows the time course of changes in cardiac function, measured as cardiac work, in control and Cytomix-treated hearts. Cardiac work in control hearts remained stable for the first hour of perfusion, followed by a gradual decline over the second hour. Cytomix-treated hearts

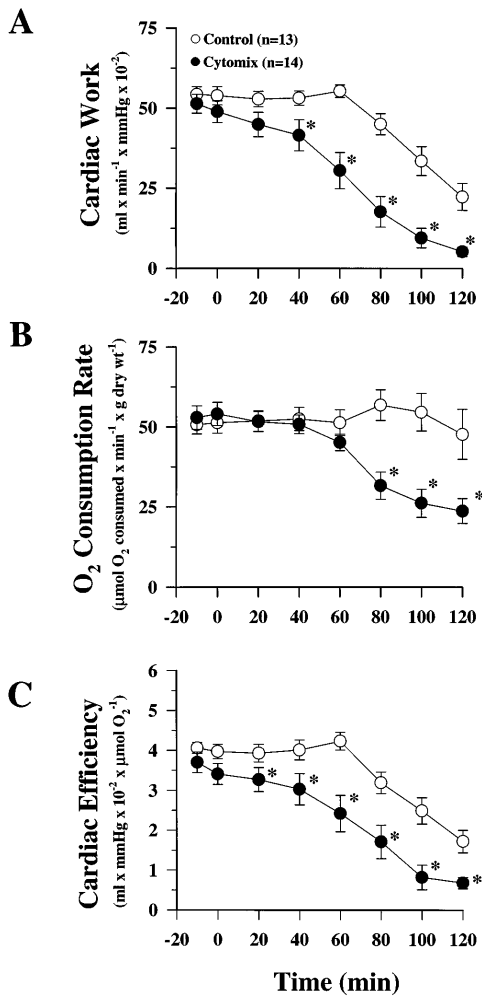


Fig. 1. Effect of interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  (Cytomix) on time course of changes in cardiac work (A), myocardial O<sub>2</sub> consumption rate (MV̇O<sub>2</sub>; B), and cardiac efficiency (cardiac work/MV̇O<sub>2</sub>; C) in rat isolated working hearts. ○, Control hearts; ●, hearts treated with Cytomix at  $t = 0$  h. \*  $P < 0.05$  vs. control, unpaired  $t$ -test.

showed a significant loss in cardiac work beginning at  $t = 40$  min of perfusion. At  $t = 2$  h, cardiac work in control hearts was  $42 \pm 8\%$  of onset values (measured at  $t = 0$  h;  $n = 13$ ,  $P < 0.05$ ) and markedly reduced in Cytomix-treated hearts ( $10 \pm 3\%$ ,  $n = 14$ ,  $P < 0.05$  vs. control at  $t = 2$  h). O<sub>2</sub> consumption rates (Fig. 1B) were not significantly altered throughout the 2 h of perfusion of control hearts, whereas they were significantly depressed in Cytomix-treated hearts beginning at  $t = 80$  min of perfusion and remained significantly depressed until the end of perfusion to  $46 \pm 7\%$  of onset values ( $P < 0.05$ ). Expressed as the ratio of cardiac work to O<sub>2</sub> consumption rates, cardiac efficiency (Fig. 1C) in control hearts was stable for the first 60 min and then gradually declined during the second hour of perfusion. In marked contrast, Cytomix caused a significant early reduction in cardiac efficiency within 20 min that continued to decline for the duration of perfusion.

**Effects of MEG and Dex on the cardiac depressant actions of Cytomix.** MEG (1  $\mu$ M) or Dex (3  $\mu$ M) abolished the enhanced loss in cardiac work induced by

Cytomix treatment (Fig. 2A) and the delayed reduction in MV̇O<sub>2</sub> (Fig. 2B). Both MEG and Dex abolished the early and late phases in the decline in cardiac efficiency stimulated by Cytomix treatment (Fig. 2C).

**Effects of MEG and Dex on hearts perfused without Cytomix.** Additional hearts were perfused without cytokines, and the actions of MEG (1 or 30  $\mu$ M) or Dex (3  $\mu$ M) were compared with the response in control hearts (Fig. 3). Neither 1 nor 30  $\mu$ M MEG reduced the spontaneous loss in cardiac work seen during the second hour of perfusion, and at neither low nor high concentration was the cardiac work profile statistically different from that of control hearts (Fig. 3A). A modest but statistically insignificant improvement in cardiac work during the 2-h perfusion time was observed in Dex-treated hearts. MEG or Dex did not significantly alter O<sub>2</sub> consumption rates (Fig. 3B) or cardiac efficiency (Fig. 3C) in the absence of cytokines.

**Effect of treatments on coronary flow and conductance.** As shown in Table 1, coronary flow in all heart groups was not significantly different at  $t = 0$  h ( $P > 0.05$ , 1-way ANOVA). At  $t = 1$  h, coronary flow was significantly depressed in Cytomix-treated hearts only,

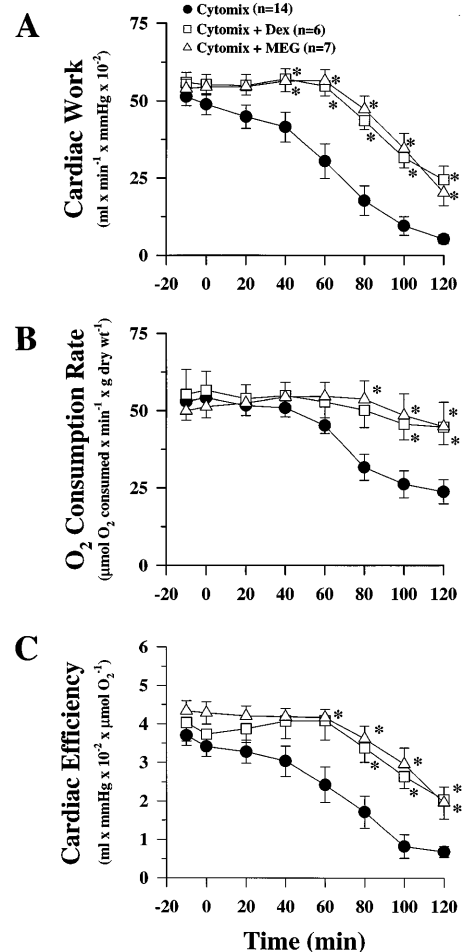


Fig. 2. Time course of changes in cardiac work (A), MV̇O<sub>2</sub> (B), and cardiac efficiency (C) in rat isolated working hearts treated with Cytomix at  $t = 0$  h. ●, Cytomix-treated hearts; □, Cytomix + dexamethasone (Dex) (3  $\mu$ M); △, Cytomix + mercaptoethylguanidine (MEG, 1  $\mu$ M). \*  $P < 0.05$  vs. Cytomix, ANOVA.

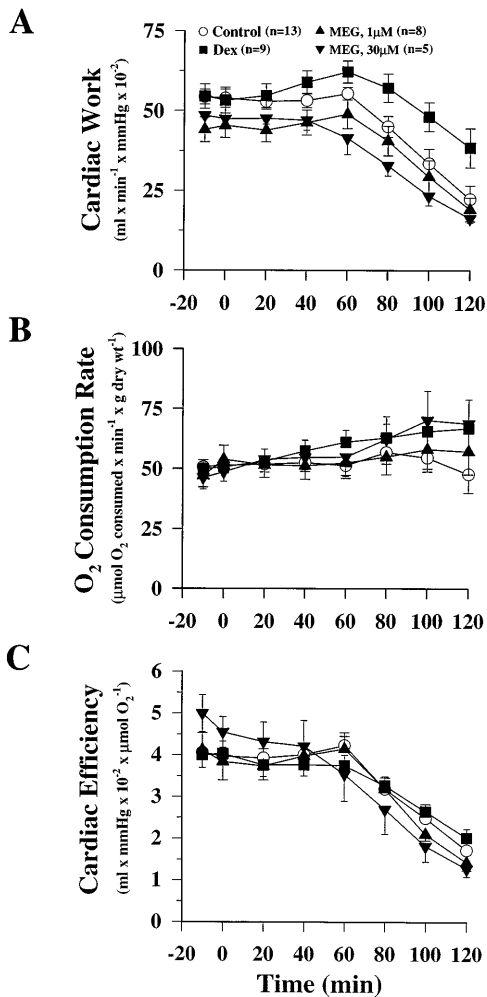


Fig. 3. Time course of changes in cardiac work (A),  $\dot{M}V_{O_2}$  (B), and cardiac efficiency (C) in rat isolated working hearts perfused without Cytomix.  $\circ$ , Control hearts;  $\blacksquare$ , Dex (3  $\mu$ M);  $\blacktriangle$ , MEG (1  $\mu$ M);  $\blacktriangledown$ , MEG (30  $\mu$ M).

whereas at  $t = 2$  h, it was significantly depressed in control, Cytomix, and Cytomix + Dex groups. To assess whether the reduction in coronary flow resulted from vasoconstriction or only reflected the decline of cardiac work, coronary conductance was also calculated (Table 2). Coronary conductance was significantly reduced at  $t = 1$  h and was further depressed at 2 h in Cytomix-

Table 1. Effects of treatment protocols on coronary flow

	n	Coronary Flow, ml/min		
		0 h	1 h	2 h
Control	13	23.7 $\pm$ 1.4	22.9 $\pm$ 1.9	18.5 $\pm$ 2.9*
Dex (3 $\mu$ M)	9	22.3 $\pm$ 0.9	26.0 $\pm$ 2.3	26.0 $\pm$ 3.7
MEG (1 $\mu$ M)	8	21.2 $\pm$ 2.2	20.6 $\pm$ 2.2	21.0 $\pm$ 3.0
MEG (30 $\mu$ M)	5	20.6 $\pm$ 1.9	21.6 $\pm$ 2.9	22.0 $\pm$ 3.3
Cytomix	14	24.8 $\pm$ 1.5	19.9 $\pm$ 1.5*	10.3 $\pm$ 1.8*†
Cytomix + MEG (1 $\mu$ M)	7	21.4 $\pm$ 0.5	22.3 $\pm$ 1.4	17.4 $\pm$ 3.0
Cytomix + Dex (3 $\mu$ M)	6	25.0 $\pm$ 2.2	22.0 $\pm$ 2.4	18.0 $\pm$ 2.4*

Values are means  $\pm$  SE. Dex, dexamethasone; MEG, mercaptoethylguanidine; Cytomix, interleukin- $1\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$ . \* $P < 0.05$  vs. 0 h; † $P < 0.05$  vs. 1 h (1-way repeated-measures ANOVA).

Table 2. Effects of treatment protocols on coronary conductance

	n	Coronary Conductance, ml $\cdot$ min $^{-1}$ $\cdot$ mmHg $^{-1}$		
		0 h	1 h	2 h
Control	13	0.39 $\pm$ 0.02	0.36 $\pm$ 0.02	0.32 $\pm$ 0.05
Dex (3 $\mu$ M)	9	0.35 $\pm$ 0.01	0.40 $\pm$ 0.03	0.39 $\pm$ 0.05
MEG (1 $\mu$ M)	8	0.35 $\pm$ 0.04	0.34 $\pm$ 0.04	0.35 $\pm$ 0.05
MEG (30 $\mu$ M)	4	0.35 $\pm$ 0.05	0.34 $\pm$ 0.04	0.35 $\pm$ 0.06
Cytomix	11	0.40 $\pm$ 0.03	0.30 $\pm$ 0.03*	0.23 $\pm$ 0.03*†
Cytomix + MEG (1 $\mu$ M)	7	0.35 $\pm$ 0.01	0.36 $\pm$ 0.02	0.28 $\pm$ 0.04
Cytomix + Dex (3 $\mu$ M)	6	0.40 $\pm$ 0.04	0.33 $\pm$ 0.04	0.27 $\pm$ 0.04*

Values are means  $\pm$  SE. \* $P < 0.05$  vs. 0 h; † $P < 0.05$  vs. 1 h (1-way repeated-measures ANOVA).

treated hearts. No changes were seen in any other groups except for the reduction in coronary conductance in the Cytomix + Dex group at  $t = 2$  h (Table 2).

**Concentration-dependent protective effect of MEG in preventing cytokine-induced myocardial depression.** The concentration dependence of MEG in reducing Cytomix-mediated cardiac depression was determined. Figure 4 shows cardiac work,  $O_2$  consumption, and cardiac efficiency after 2 h of perfusion with Cytomix in the presence or absence of MEG (0–30  $\mu$ M). Optimal protection from the Cytomix-induced loss in cardiac work and efficiency was observed with 1  $\mu$ M MEG (Fig. 4, A and C), whereas 0.3  $\mu$ M was optimal in reducing the loss in myocardial  $O_2$  production (Fig. 4B). In the presence of Cytomix, in contrast to the lack of effect of 1  $\mu$ M MEG on coronary flow (Table 1) or coronary conductance (Table 2), 30  $\mu$ M MEG significantly reduced both coronary flow (from  $21.0 \pm 0.7$  at  $t = 0$  h to  $7.3 \pm 0.9$  ml/min at  $t = 2$  h) and conductance (from  $0.32 \pm 0.02$  at  $t = 0$  h to  $0.15 \pm 0.01$  ml  $\cdot$  min $^{-1}$   $\cdot$  mmHg $^{-1}$  at  $t = 2$  h).

**Lack of acute effect of MEG on coronary flow.** In an additional series of hearts, MEG did not significantly alter coronary flow perfused in the absence of Cytomix. Cumulative addition (in 10-min intervals) of MEG caused no significant loss in coronary flow. Baseline coronary flow before addition of MEG was  $18.5 \pm 1.6$  ml/min and after 0.3, 1, 3, and 30  $\mu$ M MEG was  $19.7 \pm 1.8$ ,  $20.8 \pm 1.9$ ,  $21.2 \pm 2.3$ , and  $21.5 \pm 2.4$  ml/min, respectively ( $n = 6$ ).

**Detection of iNOS in ventricular homogenates.** Western blot analysis showed that iNOS protein was not detectable in ventricles of freshly isolated hearts perfused for 10 min as Langendorff hearts but was detectable at a low level in ventricles from control hearts or hearts perfused with Dex for 2 h as working hearts (Fig. 5). In marked contrast, Cytomix-treated hearts showed a 10-fold increase in iNOS protein expression, which was prevented by Dex (Fig. 5). Expression of iNOS protein was not impaired in hearts treated with Cytomix in the presence of 1  $\mu$ M MEG (Fig. 5). In accordance with the Western blot data, Cytomix treatment resulted in a significant increase in  $Ca^{2+}$ -independent NOS activity, which was abolished by Dex (Table 3). No significant effects of Cytomix or Dex were seen on  $Ca^{2+}$ -dependent NOS activity (Table 3).

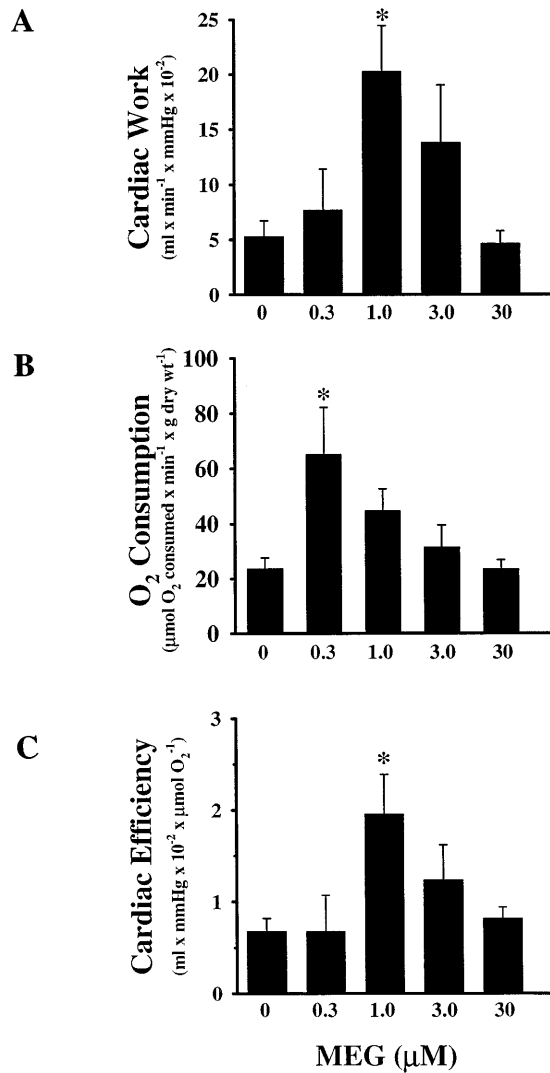


Fig. 4. Concentration dependence of protective action of MEG in Cytomix-treated hearts measured at  $t = 2$  h on cardiac work (A),  $M\dot{V}O_2$  (B), and cardiac efficiency (C). All drugs were added at  $t = 0$  h. \*  $P < 0.05$  vs. 0  $\mu$ M MEG (ANOVA).

## DISCUSSION

Our results show that the proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  caused a time-dependent and progressive depression of mechanical function in isolated working rat hearts. In marked contrast to control hearts, the depressed function was earlier in onset and progressed at a greater rate and magnitude. The loss in cardiac mechanical function is due in part to enhanced synthesis of NO as shown by the expression of iNOS protein and enhanced  $Ca^{2+}$ -independent activity in ventricular homogenates from cytokine-treated hearts and the protective actions of two chemically unrelated agents, MEG and Dex. MEG acts to preferentially inhibit iNOS compared with the other NOS isoforms (39) without affecting the expression of iNOS (Fig. 5). Dex, among a variety of pleiotropic effects, interferes with the expression of iNOS by cytokines (30). In contrast to our previous study in which hearts were exposed to IL-1 $\beta$  and TNF- $\alpha$  alone (34), we found a

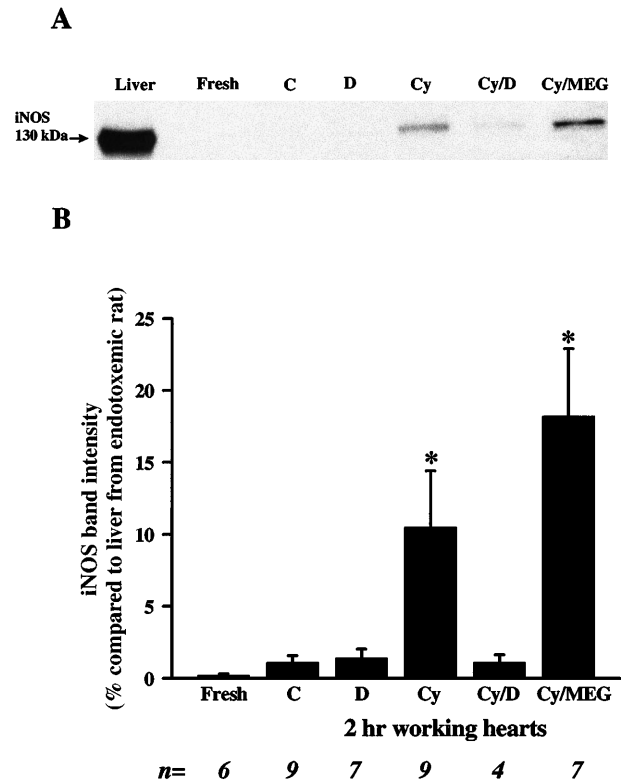


Fig. 5. A: Western blot of inducible nitric oxide synthase (iNOS) protein expression in ventricular cytosolic fractions. Liver, liver from rat 6 h after injection of 4 mg/kg endotoxin (positive iNOS control, see METHODS); Fresh, freshly isolated heart; C, control working heart after 2 h of perfusion; D, Dex-treated working heart after 2 h of perfusion; Cy, Cytomix-treated working heart after 2 h of perfusion; Cy/D, Cytomix + Dex-treated heart after 2 h of perfusion; Cy/MEG, Cytomix + MEG (1  $\mu$ M)-treated working heart after 2 h of perfusion; 90  $\mu$ g of protein per lane were loaded. B: quantitative analysis of iNOS band densities in ventricular cytosolic fractions of hearts, expressed as a percentage of iNOS band density of liver sample obtained from a rat 6 h after injection of 4 mg/kg endotoxin (see METHODS). Bar, hearts perfused for 2 h as isolated working hearts; n, no. of hearts. \*  $P < 0.05$  vs. all other groups (ANOVA).

more rapid onset (within 40 min) and enhanced rate in the depression of myocardial contractile function in hearts when IFN- $\gamma$  was added to the cytokine cocktail. IFN- $\gamma$  acts synergistically with IL-1 $\beta$  to enhance iNOS expression in cardiac myocytes (47).

Our results show for the first time that proinflammatory cytokines depress  $M\dot{V}O_2$  in the isolated working heart. Interestingly, this occurred only after 80 min of cytokine exposure, although cardiac work was signifi-

Table 3. Nitric oxide synthase activity in ventricles of hearts after 2 h of perfusion

Treatment	n	Nitric Oxide Synthase Activity, pmol $\cdot$ min <sup>-1</sup> $\cdot$ mg protein <sup>-1</sup>	
		Ca <sup>2+</sup> dependent	Ca <sup>2+</sup> independent
Control	6	1.03 $\pm$ 0.10	0.10 $\pm$ 0.04
Dex	3	0.67 $\pm$ 0.09	0.08 $\pm$ 0.04
Cytomix	6	1.36 $\pm$ 0.21	0.55 $\pm$ 0.06*
Cytomix + Dex	6	1.15 $\pm$ 0.06	0.05 $\pm$ 0.02

Values are means  $\pm$  SE. \*  $P < 0.05$  vs. control (1-way ANOVA).

cantly reduced by 40 min. Cardiac efficiency, a measure of myocardial "fuel economy," was already depressed within 20 min of cytokine treatment. This suggests that the nature of changes in the heart as a consequence of cytokine treatment are multifactorial and alter over time.

The early losses in cardiac work and efficiency may be due to a rapid release of NO in the heart (12) through transient elevation of intracellular  $\text{Ca}^{2+}$  and thus  $\text{Ca}^{2+}$ -dependent eNOS activity in endothelial cells and cardiac myocytes. That MEG was able to prevent these early changes in cardiac work and efficiency suggests that peroxynitrite, but not NO, could be mediating some of the early effects of cytokines on cardiac mechanical function, since MEG has additional actions as a peroxynitrite scavenger (41). The expression of iNOS in the heart within 20–40 min under these experimental conditions in isolated perfused hearts is unlikely, since we have previously observed a significant increase in myocardial iNOS mRNA, but not activity, 30 min after *in vivo* treatment of rats with endotoxin, whereas an increase in activity was seen at 3 h (4). This suggests that the early loss in cardiac work and efficiency by Cytomix treatment may precede the induction of iNOS. Examples for peroxynitrite generation in the absence of iNOS include hemorrhagic and endotoxin shock (42) and myocardial ischemia-reperfusion injury (52), the source of NO being eNOS. It is therefore conceivable that the protective effect of MEG against the suppression of cardiac work and efficiency in earlier time points represents an iNOS-independent, but peroxynitrite-dependent, action of the agent. This action is likely to be related to peroxynitrite scavenging and not inhibition of eNOS, since we observed no inhibition of coronary flow during acute infusion up to 30  $\mu\text{M}$  MEG. The early protective action of Dex in the present study is compatible with studies showing that superoxide generation is upregulated in rat endothelial cells particularly in response to IFN- $\gamma$  (10) and that glucocorticoids suppress increased superoxide generation in cytokine-treated cells (44). In addition, we observed a very similar loss in cardiac work and efficiency, but not  $\text{O}_2$  consumption, in isolated working hearts subjected to the continuous infusion of synthetic peroxynitrite (32), which closely mimics the picture of events during the second hour of cytokine treatment seen in this study.

NO and/or peroxynitrite have been implicated in inhibiting energy metabolic pathways in a variety of cells including smooth, skeletal, and cardiac muscle preparations (13, 14, 19, 50). Our results suggest that enhanced production of NO as a result of iNOS expression may contribute to the reduced mechanical function by interfering with mitochondrial respiration, at least in the later stages of cytokine exposure (i.e., 80–120 min). Current evidence suggests that further reaction products of NO, especially peroxynitrite, mediate the toxic actions of NO by oxidizing cellular thiols (28), inhibiting susceptible enzymes, such as aconitase (7) and mitochondrial respiratory enzyme complexes (29), and activating energy-wasting repair cycles (43).

Although cardiac mechanical function was stable for over the first 60-min period in control hearts, there was a gradual loss in cardiac work during the second hour of perfusion (Fig. 1A). The delayed onset and spontaneous loss of function in isolated working hearts has been observed by ourselves and others (45) and can be attenuated by increasing extracellular  $\text{Ca}^{2+}$  concentration (1). Our previous study with isolated working rat hearts showed that trace levels of endotoxin in the perfusion system stimulate a low-level expression of iNOS activity in the heart that is accompanied by a similar depression of mechanical function over 2 h of perfusion (34). Indeed, we observed detectable levels of iNOS protein in some hearts as a result of 2 h *in vitro* perfusion but not in freshly isolated hearts (Fig. 5). iNOS protein expression was 10-fold higher in cytokine stimulated hearts and was accompanied by a markedly enhanced cardiac depressant effect. Another distinct possibility is that the switch from *in vivo* to *in vitro* conditions is enough to stimulate the endogenous production of cytokines by the heart.

The maximal protective effect of MEG against cytokines was achieved at 1  $\mu\text{M}$ , but this effect was lost at its highest concentration (30  $\mu\text{M}$ ). Treatment of hearts with cytokines and 30  $\mu\text{M}$  MEG (but not lower concentrations of MEG), resulted in a reduction in coronary flow and conductance that was greater than that seen with cytokines alone. Moreover, the loss of protective effect at 30  $\mu\text{M}$  MEG was not due to inhibition of eNOS, since it did not reduce coronary flow in control hearts. This suggests that NO derived from iNOS activity may contribute to maintain an active vasodilator tone in the coronary circulation under stress conditions, such as sepsis. This would be particularly important as proinflammatory cytokines downregulate eNOS activity in endothelial cells (53).

In a previous study in isolated rat hearts stimulated with only IL-1 $\beta$  and TNF- $\alpha$ , the isoform nonselective NOS inhibitor L-NAME also showed a concentration-dependent action in preventing the cytokine-mediated cardiac depression (40). However, when hearts were perfused with the optimal concentration of L-NAME in the absence of cytokines, a marked loss in cardiac work due to a L-NAME-induced reduction in coronary flow took place (40). In marked contrast to L-NAME, MEG did not inhibit baseline coronary flow nor did it reduce cardiac work compared with that in control hearts, thus adding further support to the iNOS selective inhibitory profile of MEG.

Dex abolished the enhanced depression of cardiac mechanical function, the impaired  $\text{MVO}_2$ , and reduction in cardiac efficiency in cytokine-treated hearts in a similar but not equivalent fashion as MEG. However, in contrast to MEG, Dex prevented the expression of iNOS in hearts in response to cytokine treatment. Dex has previously been shown to prevent the expression of iNOS activity in isolated cardiac myocytes exposed to cytokines *in vitro* and in ventricular homogenates from rats exposed to endotoxin *in vivo* (33). The mechanisms by which glucocorticoids prevent iNOS expression by cytokines (30) are multiple. Its primary effects are

mediated by the ability of Dex to reduce iNOS mRNA translation and increase degradation of iNOS protein (24). However, other mechanisms, including actions to inhibit cytokine-stimulated superoxide generation (44), binding of nuclear factor- $\kappa$ B to the iNOS promoter (20), enhancement of lipocortin secretion (49), increased osteopontin expression (37), as well as prevention of cytokine-stimulated increase in L-arginine transport and tetrahydrobiopterin biosynthesis (36), may also mediate its actions to reduce iNOS activity or expression. Interestingly, Dex, in contrast to MEG, did not prevent the decline in coronary conductance seen in cytokine-treated hearts. This difference between Dex and MEG also further implies that some portion of NO derived from iNOS expression may help to counteract the actions of vasoconstrictor substances (i.e., catecholamines, endothelin, and eicosanoids) released in response to cytokines (21).

Increased expression of iNOS in the heart has been implicated in a variety of conditions associated with reduced cardiac mechanical function in both animals and humans, including dilated cardiomyopathies (9), allograft rejection (51), human heart failure (17), and myocardial infarct (48). Reduction in the expression of iNOS or selective inhibition of its activity prevented the enhanced deterioration of myocardial function in response to proinflammatory cytokines. Because peroxynitrite depresses cardiac work and efficiency in a concentration-dependent and irreversible manner (32), scavengers of peroxynitrite, such as MEG, should be particularly beneficial when given early after cytokine exposure. Selective inhibitors of iNOS and peroxynitrite scavengers should be tested in experimental models of heart failure to assess the functional and temporal relationship by which enhanced NO and/or peroxynitrite contribute to cardiac contractile dysfunction.

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