Glucose and pyruvate regulate cytokine-induced nitric oxide production by cardiac myocytes

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Oddis, Carmine V., and Mitchell S. Finkel. Glucose and pyruvate regulate cytokine-induced nitric oxide production by cardiac myocytes. *Am. J. Physiol.* 271 (*Cell Physiol.* 40): C1244–C1249, 1996.—Metabolic requirements for the production of nitric oxide (NO) by cytokine-stimulated neonatal rat cardiac myocytes (CM) were studied. CM were cultured for 48 h in media containing interleukin-1β (IL-1β) and free fatty acids. Removal of glucose from the media partially inhibited IL-1β-stimulated nitrite (NO_2^-) production [8.1 ± 0.3 vs. 4.4 ± 0.6 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}; $P < 0.01$; $n = 12$]. The glycolytic inhibitor 2-deoxy-D-glucose (2-DG) completely inhibited IL-1β-stimulated NO_2^- production [0.7 ± 0.5 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}; $P < 0.01$; $n = 12$]. The addition of the glycolytic end product, pyruvate, completely blocked the 2-DG inhibition of IL-1β-stimulated NO_2^- production [7.4 ± 0.4 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}; $P < 0.01$; $n = 12$]. Pyruvate alone did not significantly enhance NO_2^- production in the presence or absence of glucose ($n = 12$). The inactive analogue 3-*O*-methylglucose had no effect on NO_2^- production ($n = 12$). Reverse transcription-polymerase chain reaction revealed that pyruvate blocked 2-DG inhibition of inducible NO synthase mRNA expression. Neither 2-DG nor pyruvate had any effect on GTP-cyclohydrolase I mRNA expression in CM. We report for the first time that optimal IL-1β-stimulated NO production by CM requires both glucose and the glycolytic end product pyruvate.

cytokines; 2-deoxy-D-glucose; glycolysis; interleukin-1β; heart

NITRIC OXIDE (NO) is formed from the amino acid L-arginine by a distinct family of NO synthases (NOS) (9, 10, 12, 20). Constitutive NOSs were first described in neurons (type I) and endothelial cells (type III) (9, 10, 21, 23). Proinflammatory cytokines induce a third isoform of this enzyme (type II) in macrophages, hepatocytes, and smooth muscle cells (12, 15, 18, 24, 35). Cardiac myocytes have also been shown to constitutively produce NO and to express inducible NOS (iNOS) in response to cytokine stimulation (2, 25–27).

We have previously proposed that cytokine-stimulated NO production reversibly depresses myocardial contractility in patients with sepsis, trauma, ischemia, and congestive heart failure (1, 7, 9, 26). Cytokine-stimulated NO production has been reported to depress cardiac myocyte contractility through activation of soluble guanylate cyclase (3, 20, 29, 33). The NO-guanosine 3′,5′-cyclic monophosphate pathway has been shown to depress sarcolemmal L-type calcium channel activity and to decrease the sensitivity of contractile proteins to calcium (20, 29, 33). Thus the regulation of iNOS expression by cardiac myocytes may contribute to the pathogenesis of reversible myocardial depression.

Intracellular glucose levels and metabolic stress have been shown to regulate the expression of a number of genes (4, 14, 18). We sought to determine whether glucose plays a role in interleukin-1β (IL-1β)-induced NO production and iNOS expression in neonatal rat cardiac myocytes (CM). We report for the first time that optimal IL-1β-stimulated NO production and iNOS expression by CM require both glucose and the glycolytic end product, pyruvate.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Isolation and culture of cardiac myocytes. Myocytes were prepared from the ventricles of 1- to 2-day-old rat pups as we previously described (25, 26). Rats were decapitated immediately following CO_2 anesthesia in accordance with the guidelines of the University of Pittsburgh Animal Care and Use Committee. Briefly, the ventricles of 30–50 hearts were minced in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS, GIBCO BRL, Grand Island, NY) and digested for 15-min periods in 10 ml of a solution containing 0.1% trypsin (GIBCO BRL), 15 U/ml collagenase, and 0.1 mg/ml deoxyribonuclease (Worthington Biochemical, Freehold, NJ) in HBSS. Digestion was stopped by adding 10 ml of Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM F-12, GIBCO BRL) containing 5% calf serum. Cycles were repeated until all tissue was digested. The cell suspension was differentially plated to remove fibroblasts, endothelium, and other contaminating cell types, followed by further purification of myocytes with a Percoll gradient system in HBSS (32). The purified myocytes were cultured in DMEM-F-12 supplemented with 5% calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were seeded at a density of 1.25 × 10^5 cells/cm^2 on 48-well plates (Falcon Plastics, Cockeysville, MD; Costar, Cambridge, MA), which had been coated with a 0.05% collagen solution (Calbiochem, La Jolla, CA) and allowed to dry overnight. This preparation resulted in CM cultures with purities of ≥95%. Culture medium was changed to fresh serum-free DMEM-F-12 containing insulin, transferrin, selenium, and triglycerides containing bovine serum albumin (5.7 μg/mg, including cholesterol and linoleic acid) 48 h after plating. Myocytes formed confluent monolayers of spontaneously beating cells 24 h later. These cells were washed, and fresh serum-free DMEM-F-12 was added. IL-1β (Genzyme, Boston, MA) alone or in combination with 2-deoxy-D-glucose (2-DG) or sodium pyruvate was added at this time. Cell viability was measured by trypan blue exclusion after plating. Viability exceeded 90% and was not significantly changed by the various experimental culture conditions.

Assay for nitrite production. Nitrite (NO_2^-) assays on CM culture supernatants were performed as we have previously described (25, 26). Briefly, the stable metabolic end product of NO synthesis, NO_2^-, was used as a measure of NO production. Cell culture supernatants were mixed with an equal volume of Greiss reagent for 1 h (1 part 0.1% 1,8-naphthalenediamine dihydrochloride and 1 part 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured

with a microplate reader (Dynatech Instruments, Torrance, CA). The NO_2^- concentration was determined from a $NaNO_2$ standard curve (1×10^{-6} to 1×10^{-4} M). The absorbances from experimental supernatants were compared with known values from the standard curve to determine the NO_2^- concentrations.

Preliminary experiments were conducted to determine whether any significant nitrate (NO_3^-) as well as nitrite (NO_2^-) was produced by CM treated with IL-1β. Culture supernatants from cells treated with 500 U/ml IL-1β consistently contained a 1:3 ratio of NO_2^- to $NO_2^- + NO_3^-$, as assayed by high-performance liquid chromotography ($n = 4$). The ratio of NO_2^- to total $NO_2^- + NO_3^-$ did not change with varying experimental conditions. Thus the NO_2^- levels accurately reflected the total amount of NO produced. Only NO_2^- analyses were conducted for further experiments.

Semiquantitative reverse transcription-polymerase chain reaction. Semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR) was performed as we have previously described, with modifications (25, 26). Total cellular RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction (5). RNA was quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding 1 mM 2-deoxynucleotide-5′-triphosphate (dNTP), 1 U/μl ribonuclease inhibitor, 2.5 U/μl reverse transcriptase, and 2.5 μM oligo$(dT)_{16}$ (Perkin-Elmer, Foster City, CA) to 5 mM $MgCl_2$, 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 8.3) in a final volume of 20 μl. RT was carried out at 37°C for 1 h, followed by heat inactivation of the enzyme at 99°C for 5 min. PCR was then carried out by adding 1 μl first-strand cDNA to 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM sense (5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′) and antisense (5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′) iNOS primers (Clontech Labs, Palo Alto, CA), and 2 units of *Taq* polymerase (Perkin-Elmer) in a final volume of 50 μl. These samples were overlayed with 75 μl mineral oil and subjected to 35 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 2 min in a DNA thermal cycler (Perkin-Elmer). First-strand cDNA was also subjected to PCR with 0.4 μM β-actin-specific sense (5′-TTGTAACCAACTGGGACGATATGG-3′) and antisense (5′-GATCTTGATCTTCATGGTGCTAGG-3′) primers (Clontech) and GTP cyclohydrolase I-specific sense (5′-GGATACCAGGAGACCATCTCA-3′) and antisense (5′-TAGCATGGTGCTAGTGACAGT-3′) primers (DNA International, Lake Oswego, OR) in the same reaction conditions that were used for iNOS cDNA detection (22). These samples were subjected to 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. PCR products were run on 1.8% ethidium bromide agarose gels for 75 min and visualized by exposure to ultraviolet light. Single PCR-amplified products of the expected size were obtained for iNOS [497 base pairs (bp)], GTP cyclohydrolase I (372 bp), and β-actin (764 bp). ϕ-X174 RFDNA/*Hae* III fragments (DNA ladder) were used as DNA size markers (GIBCO).

Reaction conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction as determined by preliminary experiments. The reaction was linear to 40 cycles with use of the ethidium bromide detection method. Primer concentrations were in excess for every reaction, ensuring that logarithmic amplification of PCR products accurately reflected mRNA levels in cells. RT-PCR revealed no change in the iNOS mimic (internal control), thus confirming the absence of variability in the PCR between samples (26).

Statistical methods. Data represent the means ± SE of 12 different determinations derived from 4 wells each from 3 separate myocyte preparations of 30–50 hearts/preparation. Analysis of variance and the Student-Newman-Keuls test were used for multigroup comparisons. Values of $P < 0.05$ were considered statistically significant.

RESULTS

NO_2^- was assayed from the supernatants of CM exposed to IL-1β (500 U/ml) for 48 h in the presence or absence of glucose. IL-1β + glucose (17.5 mM) resulted in significant NO_2^- production [8.1 ± 0.3 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}; $n = 12$] (Fig. 1). CM exposed to IL-1β in the absence of glucose produced significantly less NO_2^- [4.4 ± 0.6 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}, $P < 0.01$; $n = 12$] (Fig. 1).

The glycolytic inhibitor 2-DG was used next to determine whether glycolysis was necessary for IL-1β stimulation of NO_2^- production. 2-DG inhibited NO_2^- formation by IL-1β + 17.5 mM glucose in a concentration-dependent manner (Fig. 2*A*). Maximal inhibition of IL-1β-stimulated NO_2^- production was achieved with 10 mM 2-DG [0.7 ± 0.5 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}] (Fig. 2*A*). The effects of 2-DG in the absence of glucose were also studied. One hundred micromolar 2-DG significantly inhibited NO_2^- production vs. IL-1β alone without glucose [1.1 ± 0.4 vs. 4.4 ± 0.6 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}, respectively; $P < 0.01$; $n = 12$] (Fig. 2*B*). Maximal inhibition of IL-1β-stimulated NO_2^- production was achieved with 1 mM 2-DG in glucose-free medium [0.2 ± 0.3 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}; $n = 12$] (Fig. 2*B*).

The effect of the glycolytic end product, pyruvate, on 2-DG inhibition of NO_2^- production was determined (Fig. 3). Pyruvate prevented 2-DG (10 mM) inhibition of NO_2^- production in the presence of 17.5 mM glucose in a concentration-dependent manner. Maximal NO_2^- was produced with 1 mM sodium pyruvate [7.4 ± 0.4 nmol·$(1.25 \times 10^5$ cells$)^{-1}$·48 h^{-1}; $P < 0.01$; $n = 12$] (Fig.

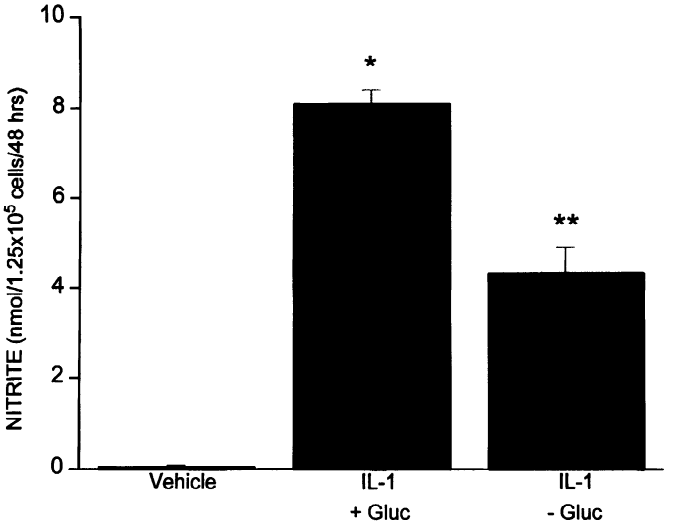

Fig. 1. Effects of interleukin-1β (IL-1β) ± glucose (Gluc) on NO_2^- production by cardiac myocytes (CM) in culture. Vehicle, 500 U/ml IL-1β + 17.5 mM glucose, or 500 U/ml IL-1β alone were added to cell cultures for 48 h, and supernatants were assayed for NO_2^-. IL-1β + glucose produced significantly more NO_2^- than vehicle ($n = 12$). *$P <$ 0.01 vs. vehicle. Removal of glucose from the culture medium significantly decreased NO_2^- ($n = 12$). **$P < 0.01$ vs. IL-1β + glucose.

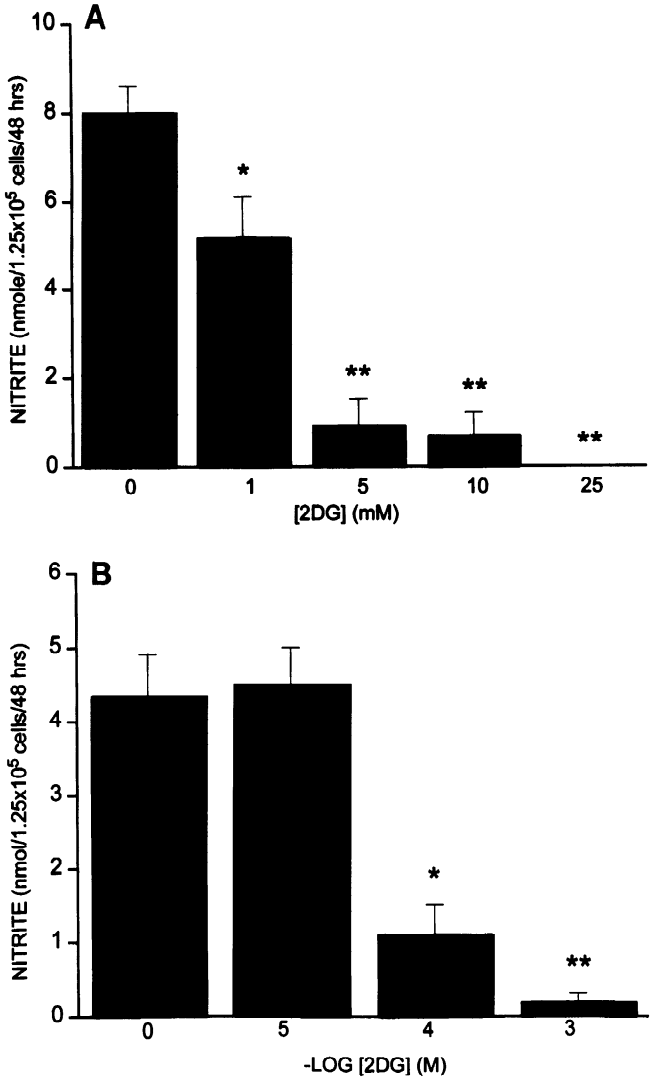

Fig. 2. Effects of IL-1β + 2-deoxy-D-glucose (2-DG) ± glucose on NO_2^- production by CM in culture. IL-1β (500 U/ml), 2-DG, and glucose (17.5 mM) were added to cell cultures for 48 h, and supernatants were assayed for NO_2^-. *A*: 2-DG (≥1 mM) inhibited IL-1β-stimulated NO_2^- production in presence of glucose. $^*P < 0.01$ vs. vehicle. $^{**}P < 0.01$ vs. 1 mM 2-DG. *B*: 2-DG (100 μM) inhibited IL-1β-stimulated NO_2^- production in absence of glucose. $^*P < 0.01$ vs. 10 μM 2-DG. $^{**}P < 0.01$ vs. 100 μM 2-DG.

3*A*). Pyruvate alone (25 mM) did not significantly increase NO_2^- production above IL-1β in the presence of glucose [8.9 ± 1.0 vs. 8.1 ± 0.3 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}, respectively; $n = 12$] or in its absence [4.8 ± 0.4 vs. 4.4 ± 0.6 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}, respectively; $n = 12$] (Fig. 3*B*). The addition of the inactive analogue, 3-*O*-methylglucose (25 mM), to IL-1β did not significantly inhibit NO_2^- production vs. IL-1β alone [8.4 ± 1.1 vs. 8.1 ± 0.3 nmol·(1.25 × 10^5 cells)$^{-1}$·48 h^{-1}; $n = 12$].

The molecular mechanisms responsible for these observations were further explored using RT-PCR (Fig. 4). Preliminary studies in CM treated with IL-1β for 24 h resulted in the expression of an RT-PCR product for iNOS mRNA (497 bp) (Fig. 4*A*), as in our previous reports (25, 26). No such product was detected in cells treated with vehicle alone (Fig. 4*A*). RT-PCR also revealed that 10 mM 2-DG inhibited IL-1β-stimulated iNOS mRNA expression (Fig. 4*A*). This inhibition was overcome by the addition of 25 mM pyruvate to CM treated with 10 mM 2-DG + IL-1β (Fig. 4*A*). RT-PCR for β-actin (764 bp) revealed no significant changes in β-actin mRNA levels in the myocytes under any of the experimental conditions (Fig. 4*A*).

GTP cyclohydrolase I is the rate-limiting enzyme for the de novo synthesis of tetrahydrobiopterin (BH_4), a critical cofactor for iNOS activity (1, 22). RT-PCR

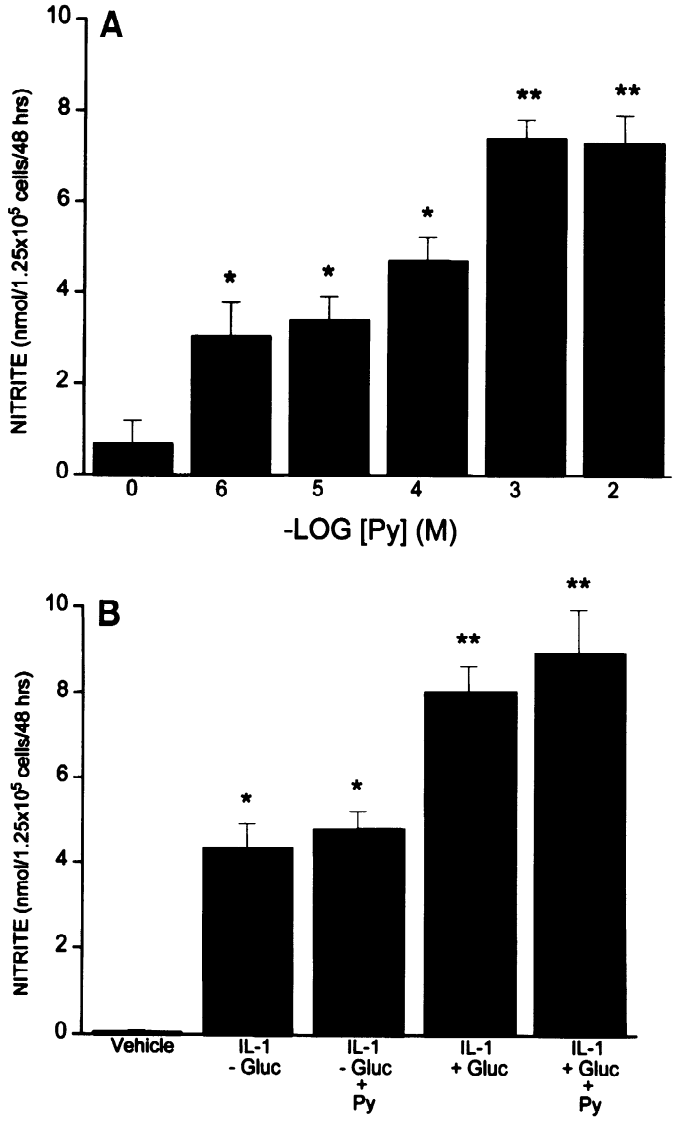

Fig. 3. Effects of IL-1β + 2-DG + pyruvate (Py) in the presence and absence of glucose on NO_2^- production by CM in culture. *A*: IL-1β (500 U/ml), 2-DG (10 mM), glucose (17.5 mM) and pyruvate were added to cell cultures for 48 h, and supernatants were assayed for NO_2^-. Pyruvate blocked 2-DG inhibition of IL-1β-stimulated NO_2^- production. $^*P < 0.01$ vs. 0 mM pyruvate. $^{**}P < 0.01$ vs. 10^{-4} M pyruvate. *B*: IL-1β (500 U/ml), glucose (17.5 mM) and pyruvate (25 mM) were added to cell cultures for 48 h and supernatants were assayed for NO_2^-. Glucose significantly increased IL-1β-stimulated NO_2^- production vs. IL-1β alone ($P < 0.01$, $n = 12$). Pyruvate did not significantly increase NO_2^- production in the presence or absence of glucose ($n = 12$). $^*P < 0.01$ vs. vehicle. $^{**}P < 0.01$ vs. IL-1β − glucose + pyruvate.

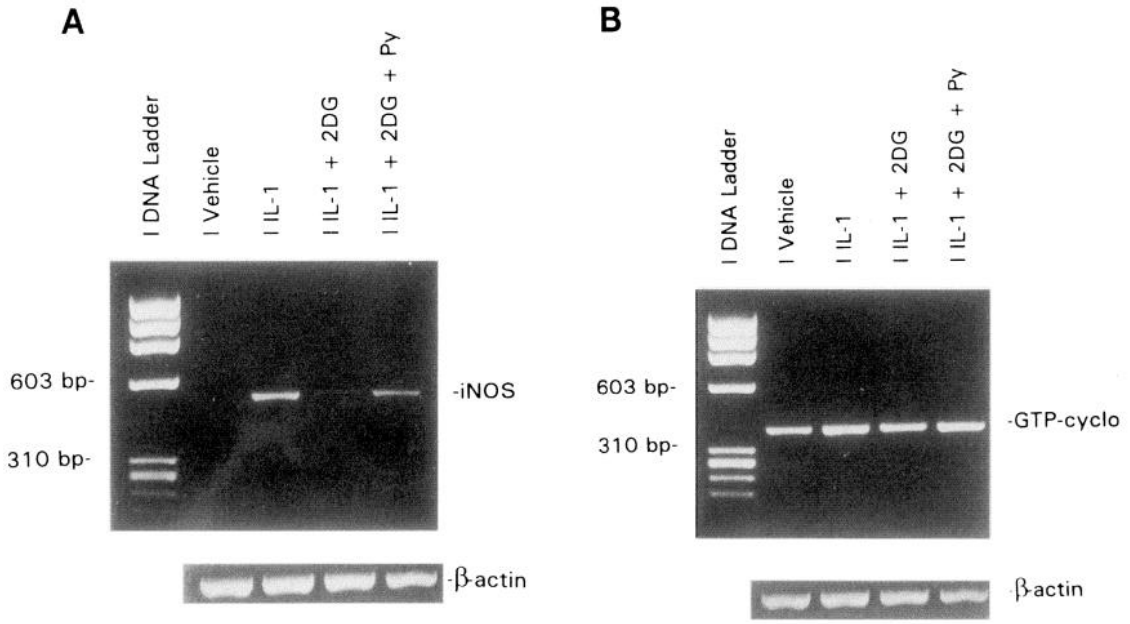

Fig. 4. Reverse transcription-polymerase chain reaction (RT-PCR) analyses of the effects of vehicle, 2-DG, and pyruvate on IL-1β-stimulated inducible nitric oxide synthase (iNOS) mRNA (*A, top*), GTP cyclohydrolase I (GTP-cyclo) mRNA (*B, top*), and β-actin mRNA (*A* and *B, bottom*) abundance in CM. Representative photographs of RT-PCR products from cells treated with vehicle, 500 U/ml IL-1β, 500 U/ml IL-1β + 10 mM 2-DG or 500 U/ml IL-1β + 10 mM 2-DG + 25 mM pyruvate for 24 h. Each experiment was repeated 3 times on 3 different CM cultures, with the same results.

revealed that cells treated with vehicle, IL-1β, and IL-1β + 10 mM 2-DG or IL-1β + 10 mM 2-DG + 25 mM pyruvate all expressed GTP cyclohydrolase I mRNA, as evidenced by the appropriate RT-PCR product (372 bp) (Fig. 4*B*). Again, RT-PCR for β-actin (764 bp) revealed no significant changes in β-actin mRNA levels in the CM (Fig. 4*B*).

DISCUSSION

The addition of IL-1β to CM for 48 h resulted in the production of detectable levels of NO_2^- in the supernatant (Fig. 1), as we previously reported (25, 26). The removal of glucose from the media resulted in a decrease in NO_2^- production (Fig. 1). The modulation of cytokine-stimulated NO production by glucose in CM is similar to the reported effect of glucose on pancreatic islet endothelial cells. NO production by islet endothelial cells but not aortic endothelial cells was modulated by glucose (31). These results suggest that glucose regulation of NO production is more organ specific than cell type specific. This may relate to the physiological importance of glucose in regulating insulin secretion in the pancreas. The present study is the first demonstration of glucose modulation of NO production by a nonendothelial cell in a nonendocrine organ.

Inhibition of glycolysis with 2-DG completely inhibited IL-1β-induced NO production (Fig. 2*A*). These results suggest that glycolysis is necessary for IL-1β-stimulated NO production by CM. Lower concentrations of 2-DG were used to preclude toxic effects (Fig. 2*B*). One hundred micromolar 2-DG was found to significantly inhibit NO_2^- production in the absence of glucose (Fig. 2*B*). Higher concentrations of 2-DG presumably were necessary to compensate for the high concentration of glucose in the media (17.5 mM). 2-DG competes with glucose for transport into cardiac myocytes (18). Much lower concentrations of 2-DG were necessary when glucose was eliminated from the media.

Studies with pyruvate provide further support for an obligatory role for glycolysis in NO production by CM. Pyruvate restored NO_2^- production by CM treated with 2-DG (Fig. 3*A*). This result is most consistent with a reversible effect of 2-DG on glycolysis that was overcome by the glycolytic end product, pyruvate. The effects of pyruvate on NO_2^- production were only apparent when cells were treated with 2-DG. The addition of pyruvate alone (without 2-DG) to IL-1β-stimulated cells in the presence or absence of glucose did not significantly increase NO_2^- production (Fig. 3*B*). These results suggest that the end product of glycolysis, pyruvate, is not sufficient for IL-1β-induced NO production. In addition, the exogenously administered pyruvate may not adequately replace intracellular pyruvate, resulting in the failure of pyruvate to reverse the glucose free effect (Fig. 3, *A* and *B*). Nevertheless, glucose appears to be required in addition to its role as substrate for pyruvate formation. NO_2^- production in the absence of glucose presumably results from the use of intracellular glycogen stores as a source for glucose (Figs. 1 and 3*B*).

We next explored potential molecular mechanisms by which metabolic stress (induced by 2-DG) could alter IL-1β-induced NO_2^- production. 2-DG decreased IL-1β-stimulated iNOS mRNA levels (Fig. 4*A*). These results are in sharp contrast to the effects of 2-DG on the

expression of heat shock and other acute phase proteins (14, 18). In those studies, 2-DG inhibition of glycolysis typically induced heat shock protein gene expression in cardiac myocytes (14). 2-DG has also been shown to inhibit gene expression of other proteins (6, 34). Pyruvate blocked 2-DG inhibition of iNOS mRNA expression in CM (Fig. 4*A*). This is most consistent with an obligatory role for glycolysis in cytokine-stimulated NO production. β-Actin mRNA levels remained unchanged throughout all of the experimental conditions to which the CM were exposed (Fig. 4*A*). This argues against nonspecific toxic effects of 2-DG.

iNOS activity has been shown to be regulated by the availability of one of its cofactors, BH_4 (2, 22). GTP cyclohydrolase I is the rate-limiting enzyme in de novo BH_4 synthesis. GTP cyclohydrolase I mRNA was found to be constitutively expressed in CM (Fig. 4*B*). The addition of 2-DG and pyruvate had no effect on GTP-cyclohydrolase I mRNA expression (Fig. 4*B*). These results also argue against a general nonspecific toxic effect of 2-DG on CM gene expression. β-Actin mRNA levels again showed no significant changes throughout these experimental conditions (Fig. 4*B*). The decrease in NO_2^- production caused by the elimination of glucose or the addition of 2-DG could be a result of a general decrease in protein synthesis, including a decrease in iNOS protein synthesis. A decrease in intracellular levels of the iNOS cofactor, NADPH, could also result in a decrease in NO_2^- production. These explanations are unlikely due to the presence of fatty acids in the media, which are the primary source of fuel in cardiac myocytes (8). Further experiments are needed to determine if protein synthesis and NADPH levels are changed under these experimental conditions.

2-DG inhibition of iNOS mRNA expression could result from a decrease in the transcription rate or message stability. Molecular studies of the mouse iNOS gene revealed that the 3′ noncoding region of iNOS mRNA contains the "AUUUA" motif known as a selective mRNA-destabilizing sequence (30). iNOS mRNA stability previously has been shown to be modulated by intracellular second messengers in cardiac myocytes, renal mesangial cells, and smooth muscle cells (15, 17, 26). The iNOS gene could also be under the control of a previously undescribed glucose-response element (18). In addition, the culture conditions could influence the effects of glucose, 2-DG, and pyruvate on NO_2^- production. For example, different insulin and serum concentrations in culture media have been shown to affect glucose uptake and utilization (11, 28). Further physiological, molecular, and biochemical studies are needed to determine which mechanism(s) is involved. Nevertheless, these findings reveal for the first time that optimal cytokine-induced NO production and iNOS mRNA expression by CM require both glucose and the glycolytic end product, pyruvate.

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