

# Acute Hyperglycemia Induces Nitrotyrosine Formation and Apoptosis in Perfused Heart From Rat

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**This study investigated coronary perfusion pressure, nitric oxide (NO) and superoxide production, nitrotyrosine (NT) formation, and cardiac cell apoptosis in isolated hearts perfused with high glucose concentration. Coronary perfusion pressure; NO and superoxide anion generation; immunostaining for NT, inducible NO synthase (iNOS), and the constitutive type of NO synthase (NOS) eNOS; iNOS and eNOS mRNA expression by Western blot and RT-PCR; and apoptosis of cardiac cells were studied in hearts perfused for 2 h with solutions containing D-glucose at a concentration of 11.1 mmol/l (control), D-glucose at the concentration of 33.3 mmol/l (high glucose), or D-glucose (33.3 mmol/l) plus glutathione (0.3 mmol/l). Perfusion of isolated hearts in conditions of high glucose concentration caused a significant increase of coronary perfusion pressure ( $P < 0.001$ ) and an increase of both NO and superoxide generation. However, superoxide production was 300% higher than baseline, whereas NO production was 40% higher ( $P < 0.001$  for both). This effect was accompanied by the formation of NT, and an increase of iNOS expression. eNOS remained unchanged. At the end of the experiments, cardiac cell apoptosis was evident in hearts perfused with high glucose. The effects of high glucose were significantly prevented by glutathione. This study demonstrates that high glucose for 2 h is enough to increase iNOS gene expression and NO release in working rat hearts. Upregulation of iNOS and raised NO generation are accompanied by a marked concomitant increase of superoxide production, a condition favoring the production of peroxynitrite, a powerful pro-oxidant that can mediate the toxic effects of high glucose on heart by itself and/or via the formation of nitrotyrosine, as suggested by the detection of cell apoptosis. *Diabetes* 51:1076–1082, 2002**

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CPP, coronary perfusion pressure; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, NO synthase; NT, nitrotyrosine;  $O_2^-$ , superoxide radical.

**C**ardiovascular complications of diabetes commonly lead to considerable morbidity and mortality (1). Recent prospective studies indicate that long-term glycemic control of diabetes is an important predictor not only of microvascular disease (2) but also of macrovascular complications, including coronary heart disease (3).

Development of stress hyperglycemia during an acute cardiovascular event is associated with a worse prognosis in patients with or without diabetes (4–7). A recent meta-analysis of published studies shows that the risk of in-hospital death in patients presenting with myocardial infarction is related to blood glucose levels at admission; this association is even more marked in patients without diabetes (8). Moreover, intensive insulin treatment during acute myocardial infarction reduces long-term mortality in diabetic patients (9). All this seems consistent with the evidence that an acute increase in plasma glucose levels in normal subjects prolongs the QT interval (10), and that hyperglycemia in acute myocardial infarction can induce such electrophysiological alterations as to favor the occurrence of life-threatening arrhythmias (11).

Among the many potential mechanisms proposed to explain why poor glycemic control may predict and worsen the prognosis of cardiovascular events in the diabetic patient, it has been reported that acute hyperglycemia can reduce nitric oxide (NO) availability, thus causing a state of increased vasomotor tone and platelet activity (12–13) and altering the adhesive properties of the endothelium (14). NO is generated from the metabolism of L-arginine by the enzyme NO synthase (NOS), of which there are three isoforms: the constitutive types bNOS (14) and eNOS (15) and the inducible type iNOS (16); the latter is induced de novo by various stimuli and leads to the production of large amounts of NO (17).

NO is inactivated by the superoxide radical ( $O_2^-$ ) (18). On the other hand, the interaction of NO with  $O_2^-$  prevents  $O_2^-$ -mediated hydroxylation reactions; thus, the activity of NO may be dependent by the local level of  $O_2^-$  generation and scavenger systems that may control vascular resistance (18). Endothelial cells can be induced to simultaneously produce NO and  $O_2^-$  in a concentrated localized manner by a variety of stimuli, including high glucose levels (19). NO and  $O_2^-$  will then react to produce peroxynitrite, a potent long-lived oxidant (20). The peroxynitrite anion is cytotoxic because it inhibits mitochondrial electron transport, oxidizes sulfhydryl groups in

protein, initiates lipid peroxidation without the requirement for transition metals, and nitrates amino acids such as tyrosine, which affects many signal transduction pathways (20). The production of peroxynitrite can be indirectly inferred by the presence of nitrotyrosine (NT) residues (21).

Because high glucose acutely increases oxygen free radical production (22) and reduces NO availability (12), the aim of the present study was to evaluate whether high glucose induces peroxynitrite generation in the heart. To test this hypothesis, coronary perfusion pressure (CPP), expression of eNOS and iNOS, production of NO and  $O_2^-$ , and generation of NT residues were evaluated in working rat hearts perfused with high or low glucose. A possible proapoptotic effect of peroxynitrite formation was also tested. Moreover, we also evaluated the effectiveness of glutathione (an antioxidant particularly active in quenching both  $O_2^-$  and peroxynitrite) (20) in counterbalancing the action of hyperglycemia.

## RESEARCH DESIGN AND METHODS

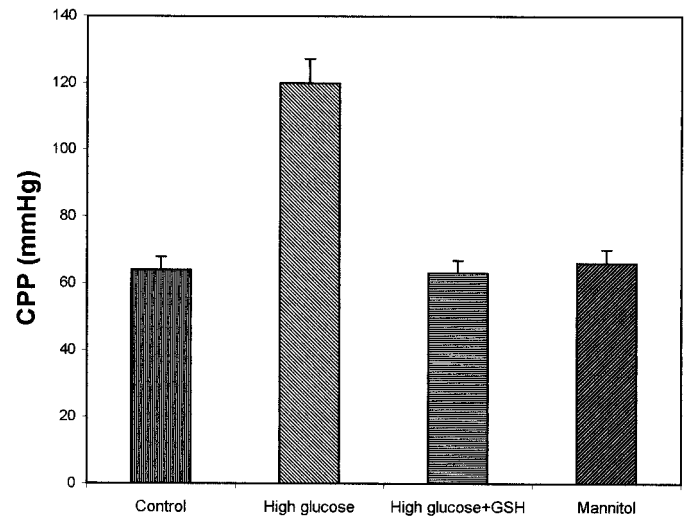
**Isolated hearts.** A total of 53 male Sprague-Dawley rats (4–6 months old and weighing on average 250 g) were anesthetized with urethane (1.2 mg/kg i.p.) and then heparinized (250 IU sodium heparin i.p., 10 min before heart excision). The hearts were rapidly excised and placed in ice-cold perfusion solution (constituents are described below) before coronary perfusion. Hearts were cannulated via the aorta and perfused retrogradely under constant flow (10 ml/min) using a calibrated roller pump (Miniplus-2; Gilson) with a buffer solution composed of the following (in mmol/l): 11.1  $D^+$ -glucose, 1.4  $CaCl_2$ , 118.5 NaCl, 25.0  $NaHCO_3$ , 1.2  $MgSO_4$ , 1.2  $NaH_2PO_4$ , and 4.0 KCl. The concentration of 11.1 mmol/l  $D$ -glucose was used as control because this concentration has been previously demonstrated to be effective in preventing hemodynamic dysfunction in this model of isolated heart (23). The buffer solution was gassed with 95% $O_2$  and 5% $CO_2$  (pH 7.4) and perfused at 37°C. Each experiment lasted 2 h.

A total of 42 hearts were used. Of these, 11 were excluded for unstable preparations. A stable preparation was defined as having a sinus rate of <220 beats per minute or a CPP >60 mmHg between 5 and 15 min after beginning of the perfusion. Any heart not in sinus rhythm during the study was also excluded. The remaining 32 hearts, which satisfied the criteria for a stable preparation, were used. Eight hearts were perfused with the buffer solution containing  $D$ -glucose at a concentration of 11.1 mmol/l, as detailed above, and these served as control hearts. Eight hearts were perfused with the buffer solution containing  $D$ -glucose at a concentration of 33.3 mmol/l (high glucose). Eight hearts were perfused with  $D$ -glucose (33.3 mmol/l) plus glutathione (0.3 mmol/l). To evaluate the effect of the osmotic solution per se, the remaining eight hearts were perfused with a buffer solution containing  $D$ -glucose (11.1 mmol/l) plus mannitol (22.2 mmol/l).

The CPP in the aortic line was monitored by a Statham Spectramed pressure transducer connected to a chart recorder (79E; Grass, Quincy, MA). Air temperature was maintained by means of a heated (37°C) water jacket. On establishing a stable CPP (20–30 min after cannulation), experiments were performed as previously described. CPP values used for statistical comparisons were calculated either as the mean of each 10 min value throughout the entire experiment or as the mean of the steady-state increment above baseline if an increase of CPP was evident during an experiment.

**Immunohistochemistry.** Rat hearts were fixed by immersion by placing only small blocks in a freshly prepared solution of 4% (wt/vol) paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 mol/l potassium phosphate buffer, pH 7.4, overnight at 4°C. The samples were then dehydrated through ascending ethanol concentrations, embedded in paraffin, sectioned (3  $\mu$ m), and mounted on slides treated with Vectabound Reagent (Vector Laboratories, Burlingame, CA).

Immunostaining was performed essentially as described previously for NT (24), eNOS (25), and iNOS (26). After deparaffinization and rehydration through decreasing ethanol concentrations, slides were immunostained using specific antibodies to NT (Upstate Biotechnology, Lake Placid, NY), iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and eNOS (Santa Cruz Biotechnology) using the Vectastain ABC Elite kit (Vector Laboratories) immunoperoxidase system. The sections were then scored for intensity of



**FIG. 1.** Coronary perfusion pressure after 2 h infusion with control (11.1 mmol/l  $D$ -glucose), high glucose (33.3 mmol/l  $D$ -glucose), high glucose plus glutathione (33.3 mmol/l  $D$ -glucose plus 0.3 mmol/l glutathione), and osmotic (11.1 mmol/l  $D$ -glucose plus 22.2 mmol/l mannitol) solutions. GSH, glutathione.

immunostaining (0 = absent, 1 = faint, 2 = moderate, and 3 = intense) for each antibody, and the average value was calculated for each section.

**Western blot analysis.** Western blot analysis was performed using specific antibodies directed against iNOS (particularly against an epitope mapping at the COOH terminus of iNOS of mouse origin), mouse, and rat-reactive, and against an epitope mapping at the COOH terminus of eNOS of human origin, mouse, rat, and human-reactive. Antibodies were purchased from Santa Cruz Biotechnology. The intensity of the Western blot signals was quantified by densitometry.

**Amplification of eNOS and iNOS mRNA by RT-PCR.** RT-PCR was performed by the use of the Superscript First-Strand Synthesis System for RT-PCR (Gibco-BRL Life Technologies).

**Measurement of NO.** NO ( $NO_2^- + NO_3^-$ ) production was evaluated by subtracting NO values at time 0 from cumulative concentrations obtained at the end of the experiments. NO was measured by the Griess' reaction, as previously described (27).

**Measurement of  $O_2^-$ .** Production of  $O_2^-$  was measured subtracting  $O_2^-$  values at time 0 from cumulative concentrations obtained at the end of the experiments, as superoxide dismutase-inhibitable reduction of cytochrome c, as previously described (28).

**Cardiac cell apoptosis.** Cardiac cell apoptosis was detected by a TACS in situ Apoptosis Detection Kit (R & D Systems Europe, Oxon, U.K.) and by Western blotting for caspase 3 (29). Antibodies for the Western blot were purchased from Santa Cruz Biotechnology.

**Statistical analysis.** Data are given as the means  $\pm$  SD. One-way ANOVA, followed by Duncan's multiple range test, and the Mann-Whitney  $U$  test were used to statistically analyze the data. A probability level of <5% was considered to be statistically significant.

## RESULTS

**CPP.** In hearts perfused with the control buffer solution, CPP was  $64 \pm 3$  mmHg (Fig. 1). This value was steady during the entire perfusion time.  $D$ -Glucose (33.3 mmol/l) determined a significant increase of CPP, evident 60 min after the start of perfusion was begun and persisting until 120 min ( $120 \pm 18$  mmHg,  $P < 0.001$  vs. control study). The effect of high glucose was completely prevented by glutathione: CPP values ( $63 \pm 5$  mmHg) were not significantly different from those observed during the control study. In the mannitol study, CPP values did not differ from those observed in the control study ( $66 \pm 4$  mmHg).

**Immunohistochemistry.** When immunostaining for the NT antigen was compared, differences were found be-



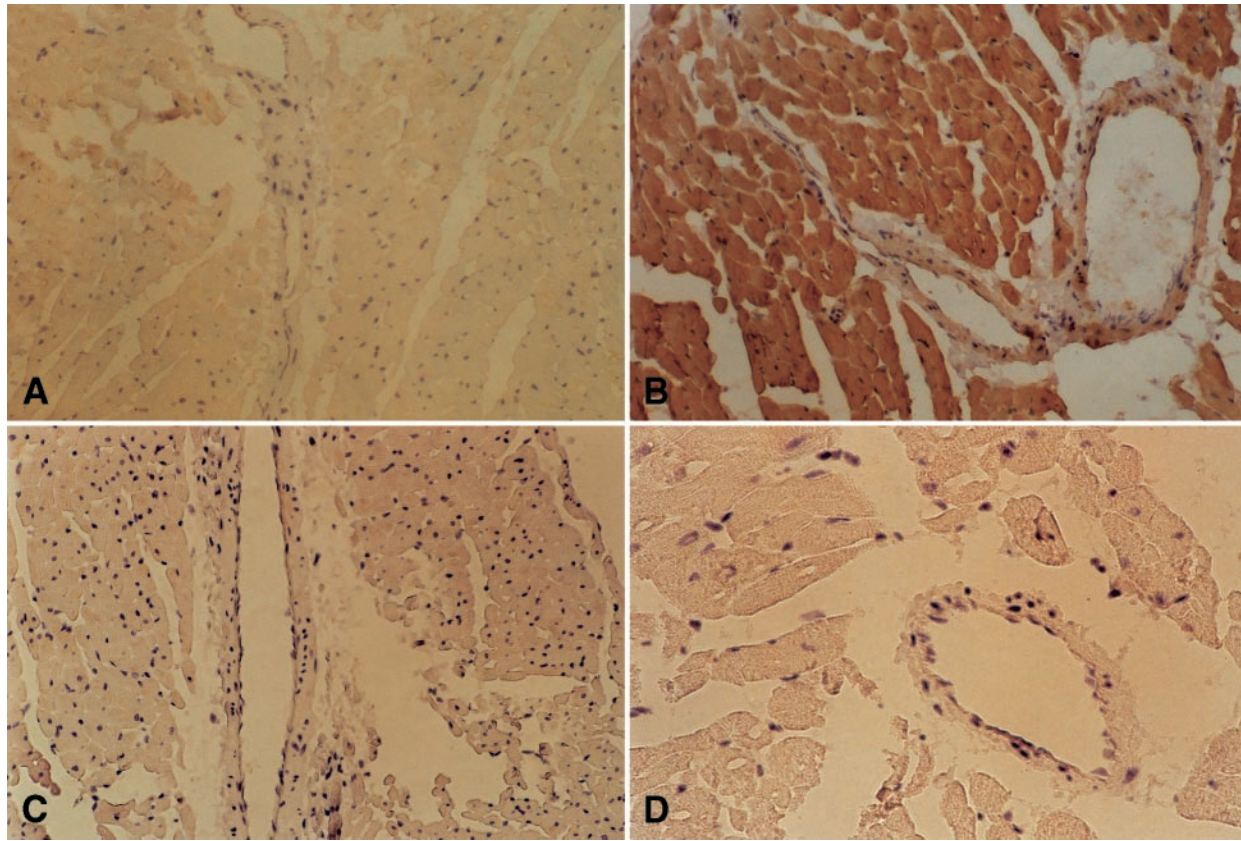


FIG. 2. Nitrotyrosine immunostaining in heart perfused with control solution (A), high glucose (B), and high glucose plus glutathione (C); negative control (D).

tween tissue from hearts perfused with high glucose and tissue from the other two groups (control and high glucose plus glutathione) (Fig. 2). In the control group, the endothelium and myocardial cells were occasionally immunostained for NT. In the absence of primary antibody, no immunostaining was apparent. In contrast, significantly intense NT immunostaining was present in tissue from hearts perfused with high glucose compared with controls (score:  $0.9 \pm 0.32$  vs.  $2.1 \pm 0.51$ ,  $P < 0.001$ ). Again, no immunostaining was seen in the absence of primary antibody. Tissue perfused with glutathione showed only a slight detection of NT. NT immunostaining of hearts perfused with high glucose and glutathione was not different from controls ( $1.1 \pm 0.41$  vs.  $0.9 \pm 0.32$ ,  $P = NS$ ), although it was significantly reduced compared with hearts perfused with high glucose alone ( $1.1 \pm 0.41$  vs.  $2.1 \pm 0.51$ ,  $P < 0.001$ ). Hearts perfused with mannitol were comparable with control hearts. NOS heart specimens perfused with high glucose, with or without glutathione, stained positive for iNOS, which was almost undetectable in controls (Fig. 3). There was a significant difference between hearts perfused with high glucose and controls (score:  $1.7 \pm 0.23$  vs.  $0.8 \pm 0.28$ ,  $P < 0.01$ ) and hearts perfused with high glucose and glutathione and controls ( $1.9 \pm 0.31$  vs.  $0.8 \pm 0.28$ ,  $P < 0.01$ ), whereas no difference was found between hearts perfused with high glucose with or without glutathione ( $1.9 \pm 0.31$  vs.  $1.7 \pm 0.23$ ,  $P = NS$ ).

The tissue of every group of hearts showed strong immunostaining for eNOS (not shown). There was no difference between groups for the intensity of eNOS (high

glucose  $1.6 \pm 0.33$ , high glucose plus glutathione  $1.7 \pm 0.31$ , control  $1.7 \pm 0.36$ ).

**Western blot.** Expression of iNOS, evaluated by Western blotting (Fig. 4), was also increased in conditions of high glucose perfusion, with or without glutathione, whereas eNOS expression was not different (data not shown).

**Amplification of eNOS and iNOS mRNA by RT-PCR.** High levels of glucose, in the presence or absence of glutathione, significantly increased iNOS mRNA expression compared with control hearts (Fig. 5). In contrast, eNOS mRNA expression was not affected (data not shown).

**NO and  $O_2^-$  production.** Both NO and  $O_2^-$  production increased during high glucose perfusion (Fig. 6). However,  $O_2^-$  production was 300% higher than baseline, whereas NO production was 40% higher ( $P < 0.001$  for both). Glutathione infusion reduced  $O_2^-$  detection in our experimental conditions, whereas NO level was not significantly affected.

**Cardiac cell apoptosis.** Cardiac cell apoptosis is illustrated in Figs. 7 and 8. Only cells from hearts perfused in high glucose showed a positive presence of apoptosis.

## DISCUSSION

Recently, much attention has been paid to the possibility that an acute increase of glycemia may be dangerous for the cardiovascular system (5–8). In fact, the presence of hyperglycemia worsens the prognosis of myocardial infarction in both diabetic and nondiabetic subjects (8). Moreover, an acute increase of glucose levels reduces NO



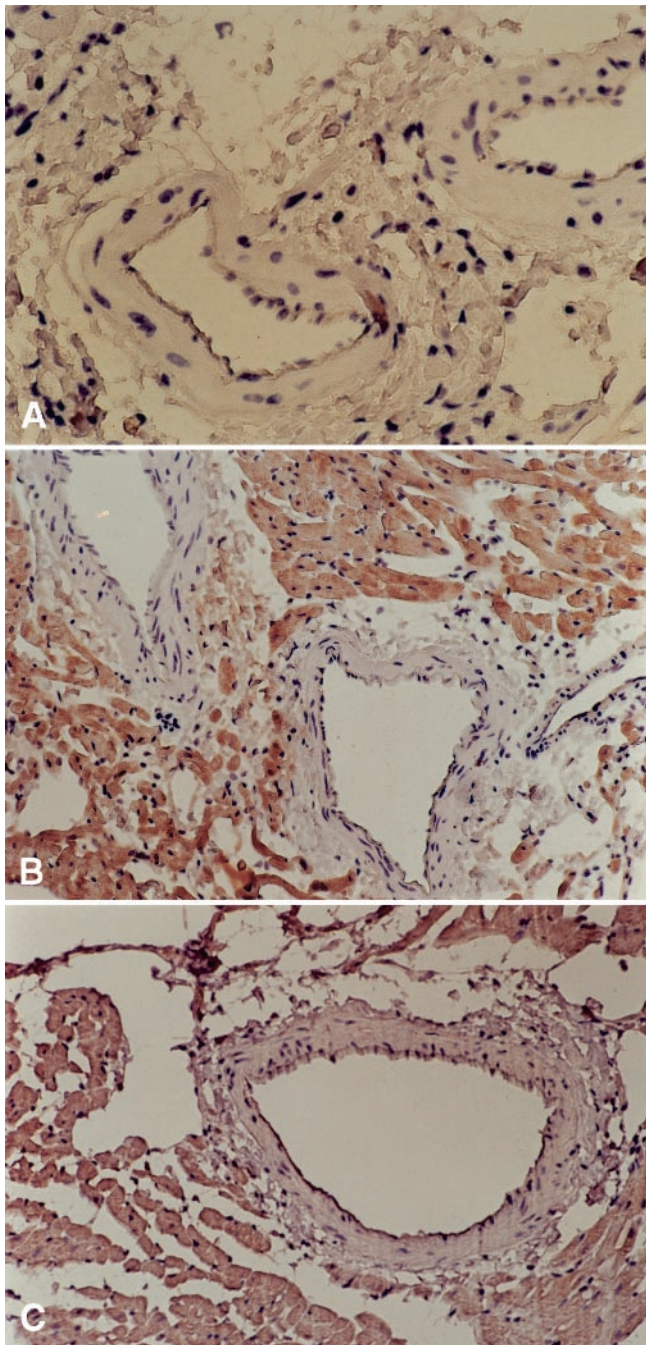


FIG. 3. iNOS immunostaining in heart perfused with control solution (A), high glucose (B), and high glucose plus glutathione (C).

availability in humans (12), through the generation of oxidative stress (30–31). In present study, high glucose concentration in the perfusion medium of isolated working rat hearts produced a significant increase of CPP, compatible with a state of increased vascular tone and hence the induction of acute endothelial dysfunction. The effects of high glucose were completely prevented by glutathione, a free radical scavenger, pointing to the mediation of increased free radical production.

Endothelial dysfunction resulting from a brief exposure to high glucose may be caused by decreased production of NO or enhanced inactivation of NO by  $O_2^-$ . This study demonstrates, for the first time, that in isolated rat

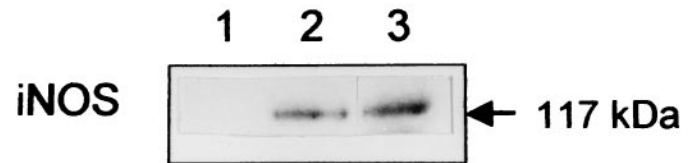


FIG. 4. Western blot of iNOS in heart perfused with control solution (lane 1), high glucose (lane 2), and high glucose plus glutathione (lane 3).

hearts, acute exposure to high glucose increases iNOS gene expression, paralleled by a simultaneous rise in both NO and  $O_2^-$  production. Accordingly, NO production increased by 40% compared with basal levels, whereas the production of  $O_2^-$  increased >300%. Thus, both synthesis and release of NO augment after acute exposure of heart to high concentration of glucose; selective upregulation of iNOS seems consistent with this conclusion.

Our findings support the hypothesis of increased NO inactivation by  $O_2^-$  as an important mechanism for the impairment of endothelial function in hyperglycemic conditions (19), and possibly for myocardial damage related to acute hyperglycemia (8). The interaction of  $O_2^-$  with NO is very rapid and leads to inactivation of NO and production of the potent oxidant peroxynitrite (21). Because NT is considered a good marker of peroxynitrite formation (21), detection of NT in hearts perfused with high glucose, but not in control hearts, is strongly suggestive for increased generation of peroxynitrite. Moreover, NT may be harmful for endothelial cells (32). The recent demonstration that the increased apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsies from diabetic patients (29) and in hearts from streptozotocin-induced diabetic rats (33) is selectively associated with levels of NT found in those cells supports our finding that high glucose may produce myocardial damage and cardiac cell apoptosis through the formation of NT.

The mechanisms by which high glucose simultaneously increases iNOS expression and  $O_2^-$  production remain to be explained, at least in part. The possibility that high glucose induces iNOS overexpression has already been reported in platelets (where this phenomenon is accompanied by increased peroxynitrite production) (34) and in

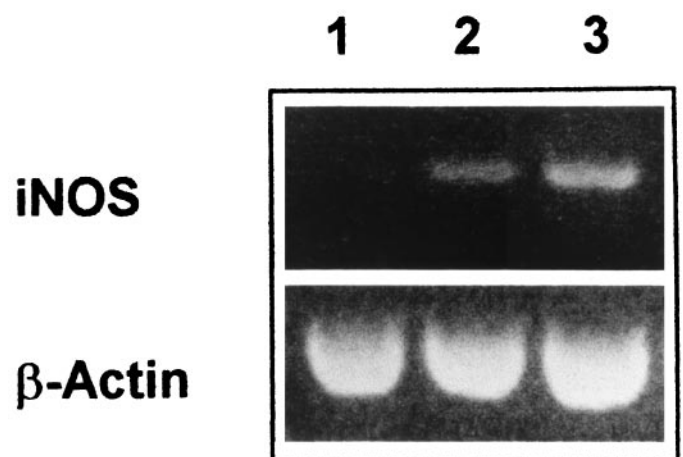


FIG. 5. mRNA of iNOS evaluated by RT-PCR in heart perfused with control solution (lane 1), high glucose (lane 2), and high glucose plus glutathione (lane 3).

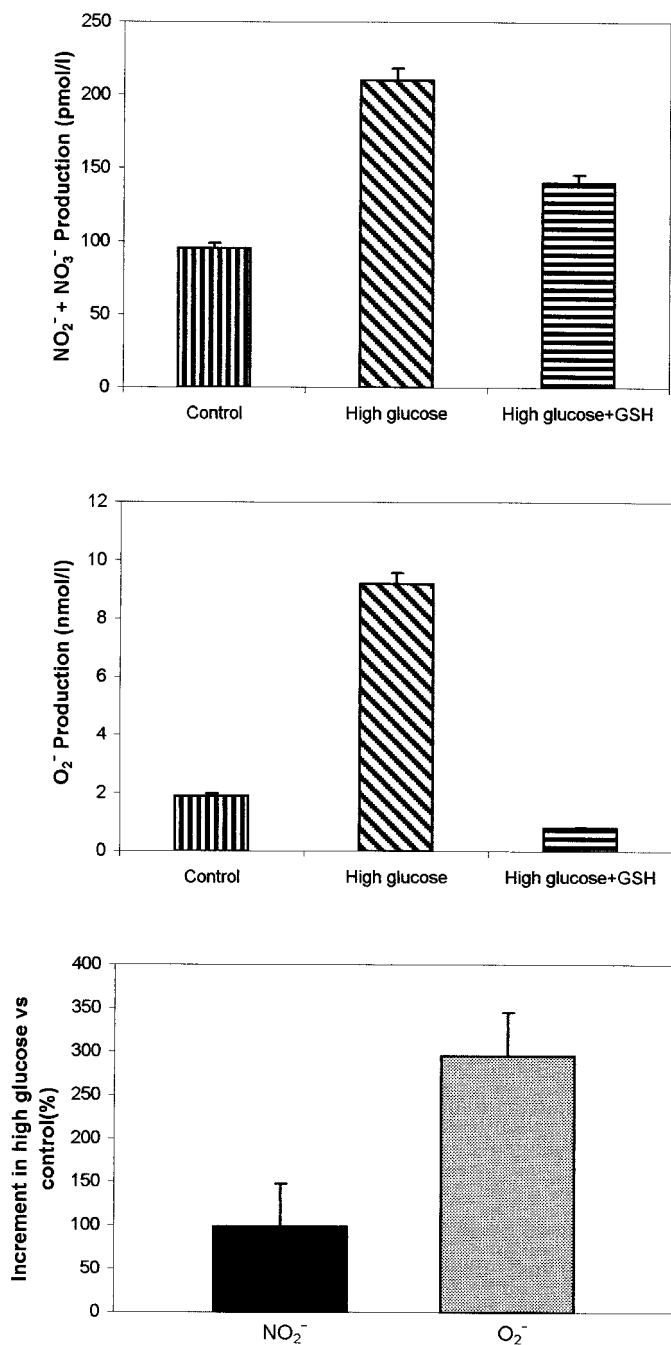


FIG. 6. NO and superoxide production during 2 h infusion in heart perfused with control solution, high glucose and high glucose plus glutathione. GSH, glutathione.

streptozotocin-diabetic rats (35). Although O<sub>2</sub><sup>-</sup> production in hearts perfused with high glucose was scavenged by glutathione treatment, iNOS mRNA and protein expression were not affected. Those results indicate that an increase in iNOS mRNA expression is not mediated, at least in the conditions of this experiments, by oxidative stress. On the other hand, it has been previously demonstrated that O<sub>2</sub><sup>-</sup> inhibition may be insufficient to inhibit iNOS expression, because iNOS expression can be induced by other factors (such as nuclear factor-κB and AP-1 activation and cytokines) that may be conditioned by hyperglycemia and that may also increase iNOS expres-

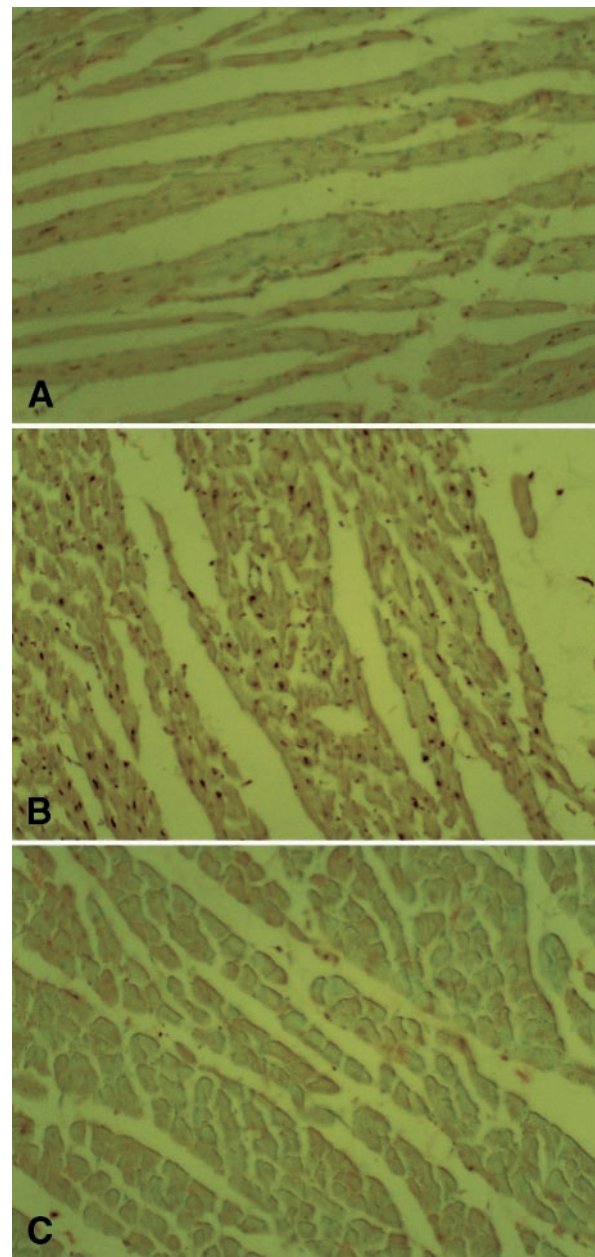


FIG. 7. Apoptosis detected in tissue from heart perfused with control solution (A), high glucose (B), and high glucose plus glutathione (C).

sion through an oxidative stress-independent pathway (36–37).

The large production of O<sub>2</sub><sup>-</sup> may be explained by the recent finding that endothelial cells are freely permeable to glucose by way of the receptor GLUT1 (38). A free glucose supply, proportional to extracellular glucose con-

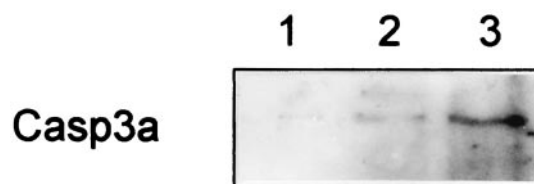


FIG. 8. Apoptosis detected by Western blot for caspase 3 (Casp3a) in tissue from heart perfused with control solution (lane 1), high glucose plus glutathione (lane 2), and high glucose (lane 3).



centration, will produce, at the mitochondrial level, not only energy but also a large quantity of  $O_2^-$  (38).

Thus, in isolated rat hearts perfused with high glucose, a parallel increase of both NO and  $O_2^-$  release occurs that is unbalanced toward a prevalent  $O_2^-$  production; in this condition, NO may be trapped by  $O_2^-$ , and peroxynitrite generation is enhanced. The important role of enhanced  $O_2^-$  production is supported by the data using glutathione, a powerful antioxidant capable of quenching both  $O_2^-$  and peroxynitrite (20). When infused along with high glucose, glutathione normalized CPP and significantly reduced NT formation in rat hearts. The similar increment of iNOS expression and NO detection in the medium observed in both conditions (high glucose versus high glucose plus glutathione) suggests that the reduction of NT formation may be explained by the quenching action of glutathione on  $O_2^-$  and/or peroxynitrite.

In summary, this study demonstrates that high glucose for 2 h is enough to increase iNOS gene expression and NO release in working rat hearts. Upregulation of iNOS and raised NO generation are accompanied by a marked concomitant increase of  $O_2^-$  production, a condition favoring the production of peroxynitrite, a powerful pro-oxidant that can mediate (by itself and/or via the formation of NT) the toxic effects of high glucose on the vascular tree. Peroxynitrite has been shown to significantly contribute to the progression of myocardial damage (39–40); the present evidence that high glucose increases peroxynitrite generation suggests that this free radical may be a mediator of the toxic effects of hyperglycemia, even during a short-time exposure, on the vascular tree.

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