Absence of Inducible Nitric Oxide Synthase Reduces Myocardial Damage During Ischemia Reperfusion in Streptozotocin-Induced Hyperglycemic Mice

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We investigated the role of inducible nitric oxide synthase (iNOS) on ischemic myocardial damage and angiogenic process in genetically deficient iNOS ($iNOS^{-/-}$) mice and wild-type littermates ($iNOS^{+/+}$), with and without streptozotocin-induced (70 mg/kg intravenously) diabetes. After ischemia (25 min) and reperfusion (120 min), both $iNOS^{+/+}$ and $iNOS^{-/-}$ diabetic mice (blood glucose 22 mmol/l) had myocardial infarct size greater than their respective nondiabetic littermates ($P < 0.01$). Myocardial infarct size ($P < 0.05$), apoptotic index ($P < 0.005$), and tissue levels of tumor necrosis factor ($P < 0.01$), interleukin-6 ($P < 0.01$), and interleukin-18 ($P < 0.01$) were higher in nondiabetic $iNOS^{-/-}$ mice compared with nondiabetic $iNOS^{+/+}$ mice. As compared with diabetic $iNOS^{-/-}$ mice, diabetic $iNOS^{+/+}$ mice showed a greater infarct size ($P < 0.01$) associated with the highest tissue levels of nitrotyrosine and proinflammatory cytokines, as well as apoptosis. The beneficial role of iNOS in modulating defensive responses against ischemia/reperfusion injury seems to be abolished in diabetic mice. *Diabetes* 53:454–462, 2004

Hyperglycemia is a risk factor for adverse outcomes during acute illness in patients with and without diabetes (1). In patients who have just experienced myocardial infarction, glucose values in excess of 110–144 mg/dl are associated with a threefold increase in mortality and a higher risk of heart failure (2). Consequently, hyperglycemia, at the time of myocardial infarction, may be an important and potentially modifiable risk factor for poor outcome. An effect of high glucose to enhance inducible nitric oxide synthase (iNOS) expression has recently been reported (3). iNOS is a calcium-independent enzyme often induced by cytokines and produces high levels of NO. Although increased NO production from iNOS may decrease vascular resistance and enhance early defensive inflammatory response against reperfusion injury (4), which are beneficial to the ischemic myocardium, high levels of NO may also depress myocardial contractility and, through formation of peroxynitrite, may cause myocardial damage (5).

Using knockout mice with a targeted disruption of the iNOS gene and control mice with a functional iNOS gene, we investigated the role of iNOS in the development of tissue damage in ischemic hearts after reperfusion during diabetes. We determined the extent of myocardial injury, apoptosis, and the levels of proinflammatory cytokines such as interleukin (IL)-18, IL-6, and tumor necrosis factor-α (TNF-α) in heart tissue.

RESEARCH DESIGN AND METHODS

Ischemia-reperfusion study was carried out in mice genetically deficient in iNOS ($iNOS^{-/-}$) and their wild-type littermates ($iNOS^{+/+}$). The homozygous $iNOS^{-/-}$ and $iNOS^{+/+}$ (wild-type C57B1/6 × 129/Sv) male mice (20–25 g; supplied by Fons A.J. Van de Loo, Department of Rheumatology, University Hospital Nijmegen, Nijmegen, The Netherlands) were generated as previously described (6). A neo cassette using homologous recombination replaced the first four exons of the NOS2 gene. All animals were allowed access to food and water ad libitum. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986). Mice were randomly allocated to one of the four following groups: $iNOS^{+/+}$ nondiabetic mice, streptozotocin (STZ) $iNOS^{+/+}$ diabetic mice, $iNOS^{-/-}$ nondiabetic mice, and STZ $iNOS^{-/-}$ diabetic mice (20 sham-operated animals and 20 infarcted animals in each group).

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STZ-treated mice. Experiments were conducted in wild-type mice and $iNOS^{-/-}$ mice (4–6 months of age and weighing on average 25 g). Under sodium pentobarbital anesthesia (50 mg/kg intraperitoneally) and aseptic conditions, a catheter was inserted into the femoral vein. This was passed subcutaneously and exteriorized on the back of the neck. Incisions were infiltrated with penicillin G procaine (300,000 IU/ml) and bupivacaine (11.25%) at closure. The venous catheter was connected to a syringe pump (Harvard Apparatus, Edenbridge, Kent, U.K.) that ran continuously throughout the study. All solutions contained antibiotic (25,000 IU of penicillin G per mouse per day and 0.03 g of meziocillin per mouse per day) and were infused through a Millipore filter (0.22-mm; Cathivex, Millipore, Bedford, MA). STZ (70 mg/kg intravenously) was administered at 4:00 P.M. through a venous catheter. In the morning of the next day, 15 h later, the venous catheter was connected to a syringe pump (Harvard Apparatus) and a continuous intravenous infusion of regular insulin (1.5 ± 0.5 units/day) was begun and adjusted to yield blood glucose levels of ~22 mmol/l (396 mg/dl) for 8 days. The other 20 wild-type and 20 $iNOS^{-/-}$ mice served as nondiabetic controls and underwent the same surgical procedures, including the intravenous catheter. Plasma glucose level was determined daily with a Glucocard Memory 2 analyzer (Menarini Diagnostics, Firenze, Italy) using ~50 μl of blood from the venous catheter.

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IL, interleukin; iNOS, inducible nitric oxide synthase; LADCA, left anterior descending coronary artery; LV, left ventricle; MABP, mean arterial blood pressure; STZ, streptozotocin; TNF-α, tumor necrosis factor-α.

Surgical procedure. The procedure described by Michael et al. (7) was essentially followed. Mice were initially anesthetized with Inactin (100 mg/kg intraperitoneally; RBI, St. Albans, U.K.) before any surgical procedure. Anesthesia was maintained via supplemental doses of Inactin (30 mg/kg intraperitoneally) as needed, and mice were prepared for coronary artery occlusion by surgical techniques. Briefly, the left jugular vein was cannulated to allow administration of further anesthetic and drugs, and a tracheotomy was performed using a polythene cannula to permit artificial ventilation (Rodent Ventilator, Basile, Comerio, Italy). The tidal volume of the respirator was set at 1.0 ml/min, with the rate set at 110 strokes/min, and supplemented with 100% oxygen. The right carotid artery was cannulated for blood pressure measurement. After an equilibration period of 20 min, a left thoracotomy was performed (between the fourth and the fifth ribs ~3 mm from the sternum) and the pericardium was removed to expose the heart. The chest walls were retracted by use of 5-0 or 6-0 silk or monofilament suture. Slight rotation of the animal to the right oriented the heart to better expose the left ventricle (LV). The left auricle was slightly retracted, exposing the entire left main coronary artery system. Ligation of the left anterior descending coronary artery (LADCA) was performed using a 7-0 silk suture attached to a 10-mm micropoint reverse cutting needle (Ethicon 7/0 BV1; Pomezia, Roma, Italy). The mean arterial blood pressure (MABP) was continuously recorded by a Mac Lab system. The heart rate was automatically calculated from blood pressure. A rectal thermometer was inserted, and the mice were kept at a body temperature of 37–38°C by a homeothermic blanket.

After completion of the surgical procedure, the animals were allowed to stabilize for 30 min before occlusion of LADCA. Both ends of the ligature around the coronary artery were threaded through a small polythene button that was placed in contact with the heart. Coronary artery occlusion was achieved by applying tension to it and clamping the ligature against the button with a small, light-weight, rubber-sheathed artery clip, without damaging the artery. After 25 min of myocardial ischemia, the clip was removed so that the tension on the ligature was released and reperfusion was allowed for 2 h.

Measurement of infarct size. Two hours after the reperfusion period, the LADCA was re-occluded, and Evans blue dye (1.5 ml of 1%) was injected retrogradely into the carotid artery catheter to delineate the area at risk. At the end of the protocol, the heart was then removed and the LV of each heart was excised and weighed. After this procedure, the heart was sectioned transversely into five sections with one section being made at the site of ligature, and the sections were weighed. Sections of the ventricle above the site of the ligature were uniformly completely blue. Sections of the ventricle from the level of the ligature to the apex, which had areas that were not blue (the area at risk) were then incubated in 1.5% triphenyltetrazolium chloride. After triphenyltetrazolium chloride staining, viable myocardium stains brick red and the infarct appears pale white. The area of infarction for each slice was determined by computerized planimetry using an image analysis software program (National Institutes of Health Image Software) as previously described by Michael et al. (7). The size of infarction was determined by the following equation: weight of infarction = (A1 × WT1) + (A2 × WT2) + (A3 × WT3) + (A4 × T4), where A is percentage of area of infarction by planimetry from subscripted numbers 1–4 representing section, and WT is weight of the same numbered sections. Percentage of infarcted LV is (WT of infarction/WT of LV) × 100. Area at risk as percentage of LV was calculated by (WT of LV − WT of LV stained blue)/WT of LV. The weight of LV stained blue was calculated in a similar manner by sum of products of the percentage of area of each slice × the weight of the respective slice (8). Infarct size analyses were performed by an unaware operator, blinded as to the group and/or strain of mouse.

Biochemical determination. Selected experiments were repeated with n = 20 mice per group. Mice were killed at the end of the ischemia. Hearts were rapidly harvested, the atria and right ventricles vessels were removed from the hearts, and the area at risk of LV was used for histological and biochemical studies. After the ischemia-reperfusion injury procedure, the LV was excised without performing any staining procedure and tissue was frozen at −80°C.

Extraction of RNA. At the end of the protocol, the heart was excised and half of each biopsy specimen was fixed in formalin, sectioned to a thickness of 5 μm, mounted on slides, and stained with hematoxylin and eosin. The mounted specimens were then examined for evidence of acute ischemia and early or evolving infarction. The other half of each specimen was frozen in liquid nitrogen at −140°C. Portions of the frozen samples were lyophilized, and then RNA was extracted by the acid guanidinium thiocyanate-phenolchloroform technique. The recovered RNA pellet was dried under vacuum conditions for 10–15 min and then dissolved in diethyl pyrocarbonate–treated deionized distilled water. The concentration and the purity of the RNA were determined by spectrophotometric analysis (Ultrospec II; Biochrom, Cambridge, England) at 260 and 280 nm. The samples were stored at −80°C until analyzed.

At the end of each experimental procedure described above, four hearts for each experimental group were dissected and total RNA was extracted from the whole heart using RNAzol reagent (Biotecx Lab) according to the manufacturer's protocol. Appropriate regions of the hypoxanthine-phosphoribosyl-transferase cDNA were amplified as control. Amplifications were carried out for 32 cycles, using the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min.

Each RT-PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in 1× TAE. Semiquantitative analysis of mRNA levels was carried out using the software associated with the Gel Doc 1000 (Biorad, Hercules, CA).

Western blot analysis. iNOS was determined by immunoblot analyses. Hearts were lysed in a buffer containing 1% Nonidet P-40 for 30 min at 4°C. The lysates were then centrifuged for 10 min at 10,000*g* at 4°C. After centrifugation, 20 μg of each sample was loaded, electrophoresed in polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. Primary antibodies to detect iNOS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used according to the manufacturer's instructions. Immunoreactive signals were detected with a horseradish peroxidase–conjugated secondary antibody (Santa Cruz) and reacted with an enhanced chemiluminescence system (Amersham Pharmacia, Uppsala, Sweden). Each Western blot was repeated at least three times.

Assessment of interleukin levels in myocardium. Mouse TNF-α, IL-6, and IL-18 levels were quantified in noninfarcted heart and in the area at risk at the end of the reperfusion period using specific ELISA Kits (R&D Systems, Minneapolis, MN). Tissue homogenates (50 μl) were assayed and compared with a standard curve constructed with 0–500 pg/mg mouse TNF-α, IL-6, and IL-18.

Cardiac cell apoptosis. Cardiac cell apoptosis was assessed by caspase-3. Myocardial caspase-3 activity was measured by cytosolic extracts prepared from heart samples by homogenization and centrifugation procedures, as described above. The caspase-3 activity was quantified spectrophotometrically by a specific colorimetric sandwich ELISA assay (R&D Systems) using the protocol recommended by the manufacturer (approximate detection limit 0.1 ng/ml).

Immunohistochemistry. Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or reactive oxygen species, was determined by immunohistochemistry as previously described (9). At the end of the experiment, the tissues were fixed in 10% (wt/vol) PBS-buffered formaldehyde and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (vol/vol) hydrogen peroxide in 60% (vol/vol) methanol for 30 min. The sections were permeabilized with 0.1% (wt/vol) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, vol/vol). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). For confirming that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mmol/l) to verify the binding specificity. In this situation, no positive staining was found in the sections, indicating that the immunoreaction was positive in all of the experiments carried out. The sections were then scored for intensity of immunostaining (0 = absent, 1 = faint, 2 = moderate, 3 = intense) for each antibody, and the average value was calculated for each section.

Statistical analysis. The statistical analysis was performed with one-way ANOVA, followed by Duncan's multiple range test. A probability level of $<5\%$ was considered to be statistically significant. Two tests were applied to compare infarct size and apoptotic index among the groups. All statistical analysis was made on IBM computers with the SOLO software package (BMDP, statistical software). All data are presented as means ± SD.

RESULTS

Ischemia-reperfusion injury

Nondiabetic mice. Compared with wild-type mice, iNOS$^{-/-}$ mice had higher MABP ($P < 0.05$). Mice were subjected to 25 min of occlusion, followed by reperfusion of the LADCA of the left coronary artery. Coronary artery occlusion produced a significant fall in MABP, which was similar in both wild-type (−15 ± 1.2%) and iNOS$^{-/-}$ mice (−16 ± 1.9%). In nondiabetic iNOS$^{+/+}$ mice, occlusion of

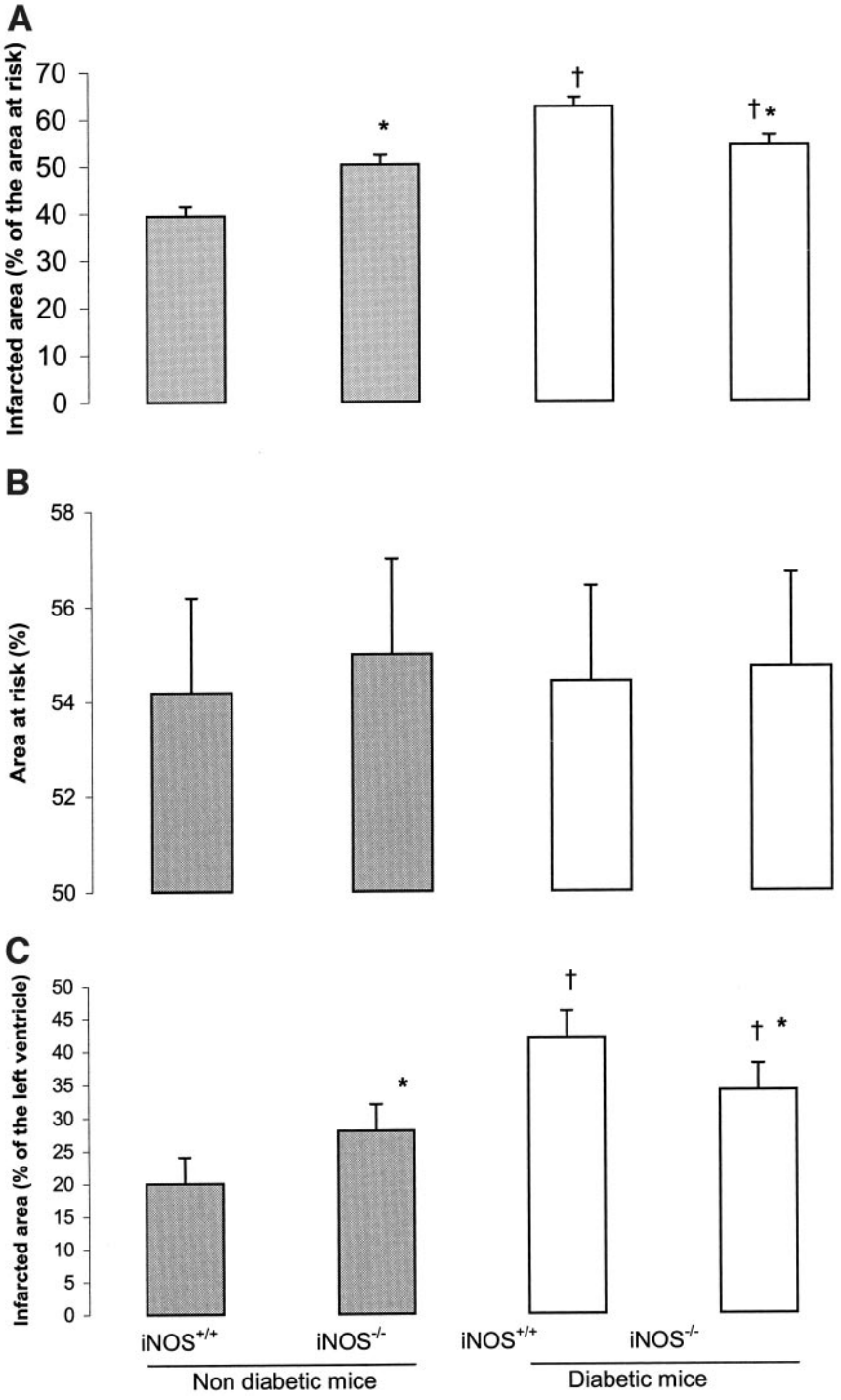

FIG. 1. Infarct size after ischemia-reperfusion in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice with and without diabetes. *A*: Infarct size, calculated as percentage of total area at risk, in iNOS$^{+/+}$ mice and iNOS$^{-/-}$ mice after myocardial ischemia (25 min) and reperfusion (120 min). *B*: Myocardial area at risk, expressed as percentage of the LV. *C*: Infarct size, calculated as percentage of the LV. Data are expressed as mean ± SD. *P < 0.05 vs. iNOS$^{+/+}$ mice; †P < 0.01 vs. nondiabetic mice.

the LADCA followed by reperfusion resulted in a mild myocardial injury (Fig. 1); the infarcted area corresponded to 39.3 ± 2.4% of the area at risk. In nondiabetic iNOS$^{-/-}$ mice, the infarcted area was 52.9 ± 2.5% of the area at risk ($P < 0.05$ vs. iNOS$^{+/+}$). Areas at risk were not different in nondiabetic iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (42.1 ± 2.9% and 45.6 ± 2.7% of total LV, respectively). The absence of a functional iNOS gene in iNOS$^{-/-}$ mice resulted in a significant augmentation of reperfusion injury of previously ischemic myocardium.

Diabetic mice. Daily blood glucose and insulin values are presented in Fig. 2. Blood glucose averaged 6.2 ± 0.1 mmol/l in basal conditions and rose to 23.3 ± 1.7 mmol/l 15 h after STZ administration in both wild-type and iNOS$^{-/-}$ mice. A continuous intravenous infusion of regular insulin (1.4 ± 0.3 units/day) was begun and adjusted to yield blood glucose levels of ~22 mmol/l (396 mg/dl) for 8 days. Serum sodium and potassium concentrations and body weight did not significantly change after STZ treatment in both wild-type and iNOS$^{-/-}$ mice. The 8-day period of hyperglycemia resulted in a significant increase of MABP in both wild-type and iNOS$^{-/-}$ diabetic mice ($P < 0.01$). The increase in MABP was less evident in iNOS$^{-/-}$ diabetic mice ($P < 0.05$). In diabetic iNOS$^{+/+}$

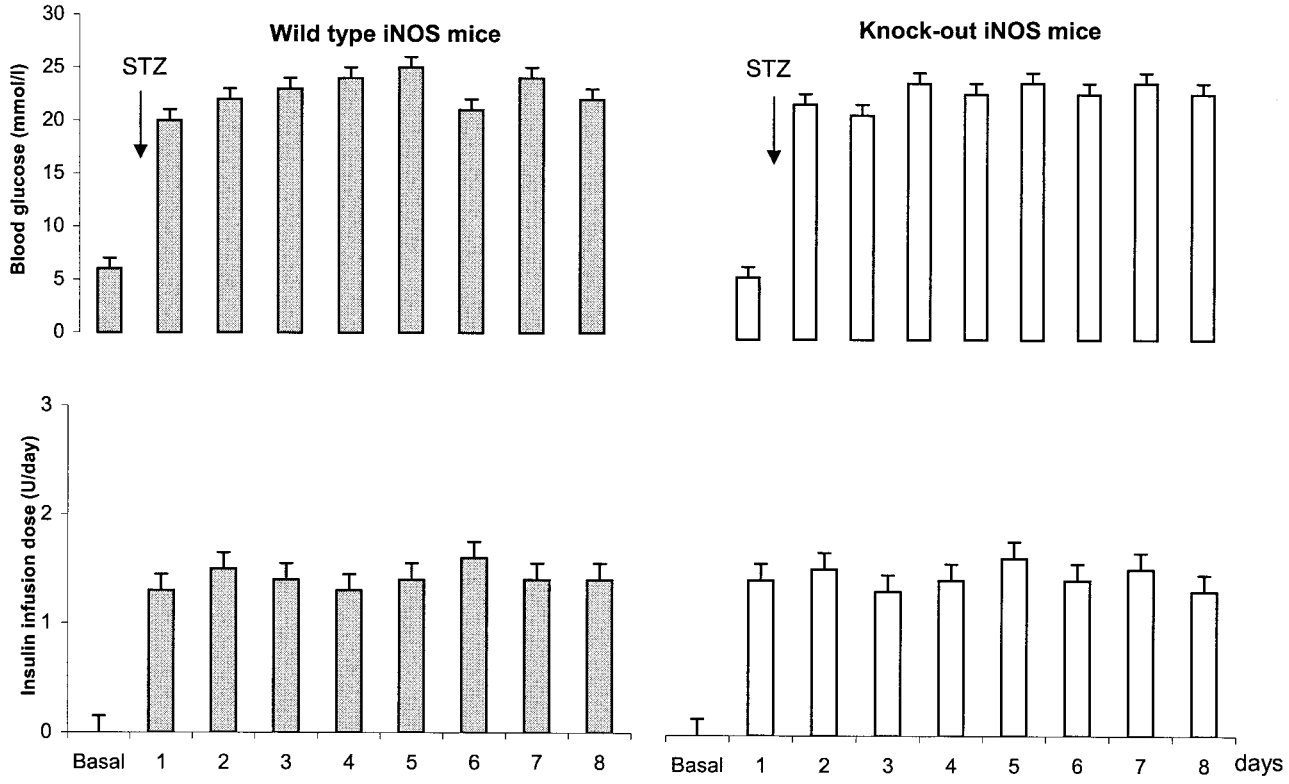

FIG. 2. Daily blood glucose levels and insulin infusion dose in diabetic mice.

mice, occlusion of the LADCA followed by reperfusion resulted in a severe myocardial injury (Fig. 1); the infarcted area corresponded to 62.5 ± 2.2% of the area at risk. In iNOS$^{-/-}$ mice, the infarcted area was 54.4 ± 2.6% of the area at risk ($P < 0.05$ vs iNOS$^{+/+}$). Areas at risk were not different in nondiabetic iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (Fig. 1). The absence of a functional iNOS gene in iNOS$^{-/-}$ mice resulted in a significant reduction of reperfusion injury of previously ischemic myocardium (Fig. 1). Coronary artery occlusion produced a significant fall in MABP, which was similar in both wild-type (−18 ± 2.3%) and iNOS$^{-/-}$ diabetic mice (−16 ± 2.1%). At the time of surgery, the serum levels of insulin and glucose were not significantly different among the groups (Fig. 2). The lipid levels were similar in both nondiabetic mice (cholesterol: iNOS$^{+/+}$ 2.3 ± 0.8 mmol/l; iNOS$^{-/-}$ 2.1 ± 0.5 mmol/l, NS; triglyceride, iNOS$^{+/+}$ 0.9 ± 0.2 mmol/l, iNOS$^{-/-}$ 1.0 ± 0.4, NS) and diabetic mice (cholesterol: iNOS$^{+/+}$ 5.3 ± 0.6 mmol/l, iNOS$^{-/-}$ 5.1 ± 0.5 mmol/l, NS; triglyceride: iNOS$^{+/+}$, 5.8 ± 0.6 mmol/l, iNOS$^{-/-}$ 5.6 ± 0.7 mmol/l). Infarct size percentage of the area at risk was significantly greater in diabetic mice, as compared with nondiabetic mice, in both wild-type ($P < 0.01$) and iNOS$^{-/-}$ mice ($P < 0.05$).

Compared with nondiabetic mice, both iNOS$^{+/+}$ and iNOS$^{-/-}$ diabetic mice had higher MABP ($P < 0.01$). Compared with iNOS$^{-/-}$ diabetic mice, iNOS$^{+/+}$ diabetic mice presented higher levels of MABP ($P < 0.05$). iNOS$^{+/+}$ diabetic mice had the highest MBP values (122 ± 3 mmHg), nondiabetic mice (104 ± 2 mmHg) had the lowest, and iNOS$^{-/-}$ diabetic mice had intermediate MABP values (116 ± 2 mmHg). Coronary artery occlusion produced a significant fall in MABP, which was similar in all groups of mice. There was no relationship between infarct size and MABP changes during occlusion and reperfusion in all groups (data not shown).

Diabetes increases cytokine production in both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. At baseline, cardiac TNF-α, IL-6, and IL-18 levels were not appreciable in both groups of nondiabetic mice but were found to be present in diabetic mice (Fig. 3). A substantial increase in TNF- α, IL-6, and IL-18 production was found in both diabetic and nondiabetic iNOS$^{+/+}$ mice after myocardial ischemia and reperfusion, which was higher in diabetic mice ($P < 0.005$). This pattern of cytokine response was also seen in iNOS$^{-/-}$ groups, although at a lower level ($P < 0.01$; Fig. 3).

Absence of iNOS reduces formation of nitrotyrosine in diabetic mice. iNOS and iNOS mRNA expressions were present in hearts from sham iNOS$^{+/+}$ groups, although at a higher level in the diabetic group ($P < 0.005$), but was absent in iNOS$^{-/-}$ animals (data not shown). iNOS and iNOS mRNA expressions increased after ischemia-reperfusion in iNOS$^{+/+}$ animals, with a further increase in diabetic mice, which was significantly higher compared with nondiabetic mice ($P < 0.005$; Fig. 4). When immunostaining for the nitrotyrosine antigen was compared, differences were found between tissues from sham iNOS$^{+/+}$ diabetic mice and sham iNOS$^{+/+}$ nondiabetic mice: intense nitrotyrosine immunostaining was present in tissue from sham iNOS$^{+/+}$ diabetic mice compared with sham iNOS$^{+/+}$ nondiabetic mouse hearts ($P < 0.001$). Nitrotyrosine staining was virtually absent in the sham iNOS$^{-/-}$ nondiabetic mice; only a slight detection of nitrotyrosine was observed in sham iNOS$^{-/-}$ diabetic mice (Fig. 4). After ischemia-reperfusion, significantly intense

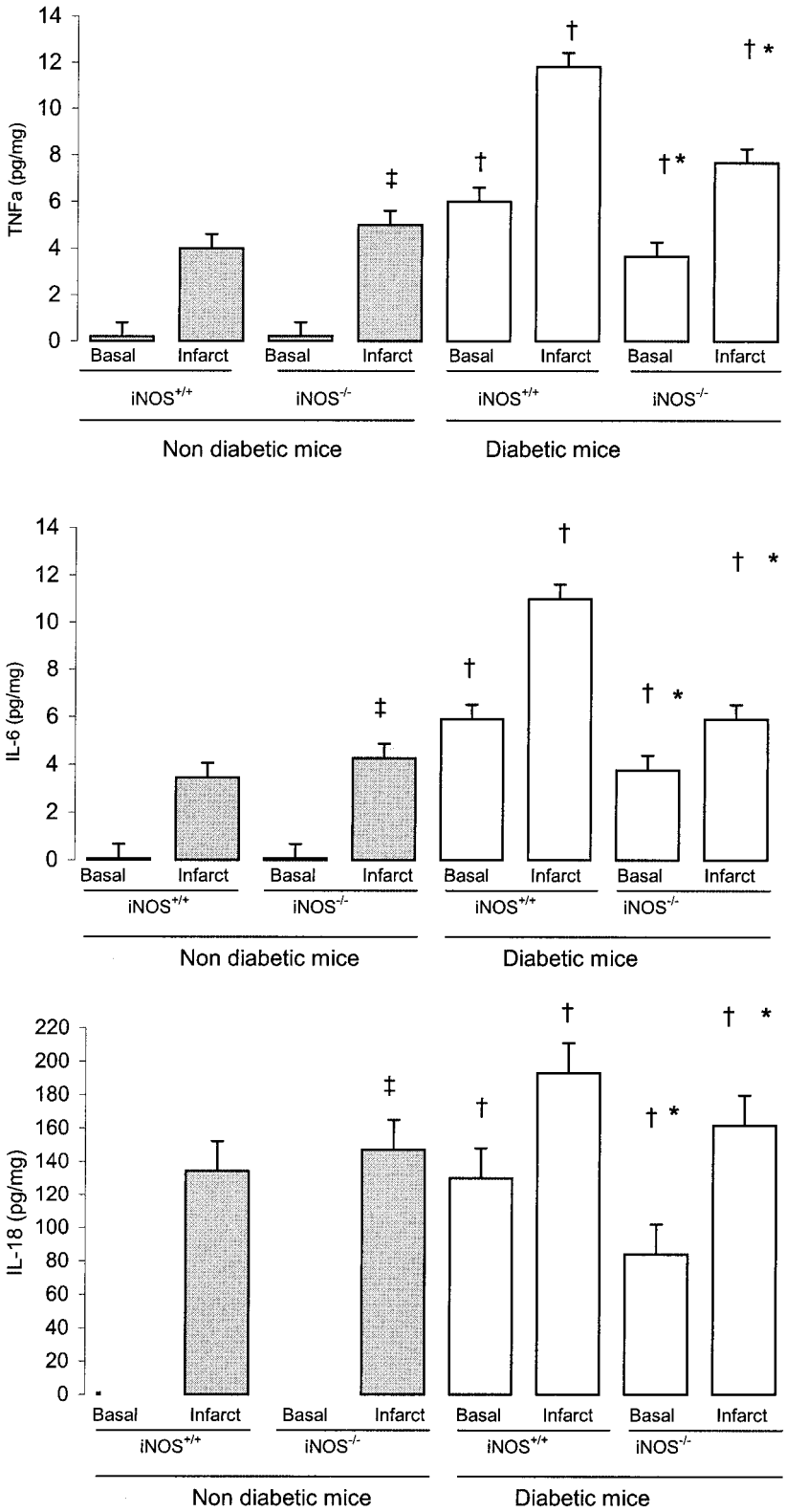

FIG. 3. TNF- α, IL-6, and IL-18 tissue levels at baseline and after ischemia-reperfusion injury in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice with and without diabetes. Values are expressed as mean ± SD. *$P < 0.005$ vs. iNOS$^{+/+}$ mice; ‡$P < 0.01$ vs. iNOS$^{+/+}$ mice; †$P < 0.005$ vs. nondiabetic mice. I/R, ischemia-reperfusion.

nitrotyrosine immunostaining was present in tissue from iNOS$^{+/+}$ nondiabetic hearts compared with iNOS$^{-/-}$ nondiabetic hearts ($P < 0.001$). Nitrotyrosine immunostaining of hearts from iNOS$^{+/+}$ diabetic mice was significantly higher compared with hearts from iNOS$^{+/+}$ nondiabetic ($P < 0.001$) and from iNOS$^{-/-}$ diabetic hearts ($P < 0.001$; Fig. 5).

Absence of iNOS increases reduces caspase-3 activity in diabetic mice. Caspase-3 activity was not different in preparations from sham iNOS$^{+/+}$ and iNOS$^{-/-}$ nondiabetic mice. Tissue homogenates from hearts of both diabetic groups showed a positive presence of caspase-3 activity that was significantly greater in iNOS$^{+/+}$ mice compared with iNOS$^{-/-}$ mice ($P < 0.05$). In diabetic mice,

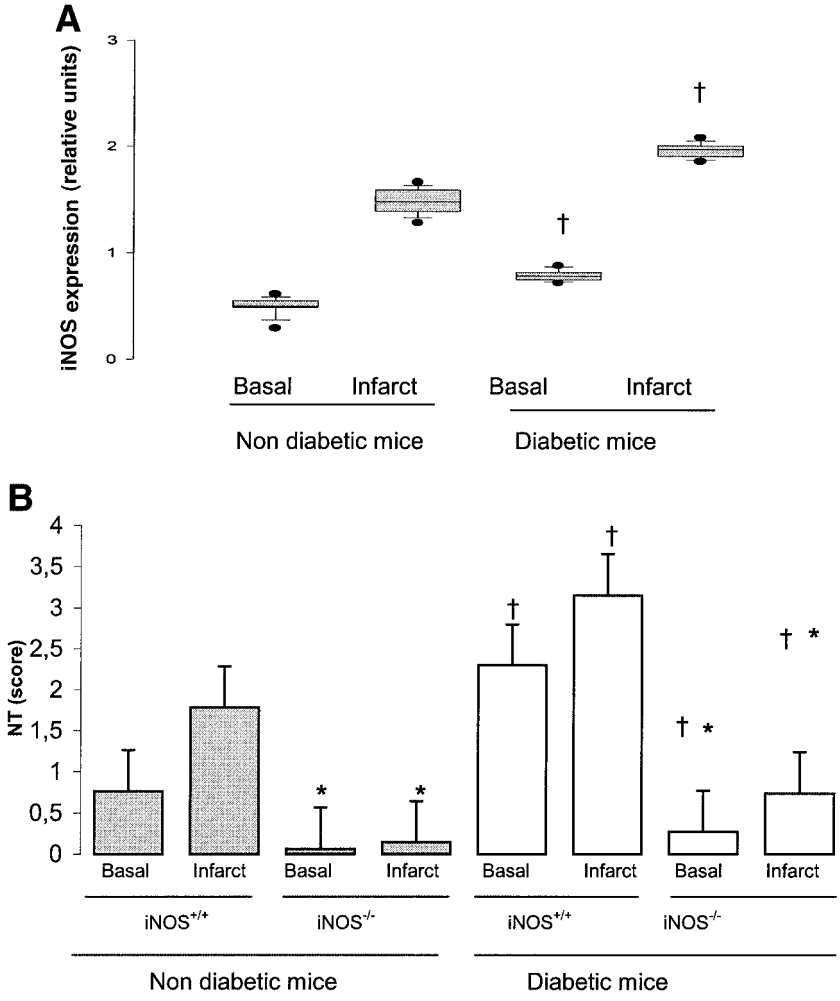

FIG. 4. ***A*: Western blot analysis of iNOS content in hearts from diabetic and nondiabetic mice at baseline and after ischemia-reperfusion injury (boxplot, a plot type that displays the 10th, 25th, 50th, 75th, and 90th percentiles as lines on a bar centered about the mean, and the 5th and 95th percentiles as error bars; the mean line and data points beyond the 5th and 95th percentiles can also be displayed).** ***B*: Nitrotyrosine levels at baseline and after ischemia-reperfusion injury in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice with and without diabetes. *P < 0.001 vs. iNOS$^{+/+}$ mice; †P < 0.001 vs. nondiabetic mice.**

the apoptotic index was higher in iNOS$^{+/+}$ mice (0.89 ± 0.05%) compared with iNOS$^{-/-}$ mice (0.56 ± 0.04%; $P <$ 0.01). In nondiabetic mice, myocardial ischemia followed by reperfusion resulted in a marked appearance of caspase activity in the left ventricular tissue preparations from iNOS$^{-/-}$ mice; only a small activity in the LV of wild-type mice ($P < 0.05$) was detected (Fig. 6). In diabetic mice, the caspase activity was higher in iNOS$^{+/+}$ mice compared with iNOS$^{-/-}$ mice ($P < 0.01$; Fig. 6).

DISCUSSION

Our data demonstrate that mice with a targeted deletion of the iNOS gene are significantly more vulnerable to pathological changes associated with myocardial ischemia and reperfusion injury than are wild-type controls, suggesting that the presence of a functional iNOS gene and the production of NO may provide cardioprotection during reperfusion injury. Despite extensive research, the role of NO in ischemia and reperfusion injury remains controversial and is yet to be defined (10,11). However, several reports demonstrated that NO functions as a protective agent during reperfusion injury. In isolated perfused Langendorff preparations, for example, a rapid superinduction of iNOS during early reperfusion attenuated a hyperdynamic response (11). Moreover, approaches to remove NO by pharmacological or genetic inhibition of iNOS have also been shown to exacerbate reperfusion injury in the heart (12), whereas approaches to deliver NO by donors have been shown to ameliorate tissue damage (13,14).

In STZ-induced diabetic mice, the picture is different because the deletion of the iNOS gene improves cardiac outcome. So the beneficial role of iNOS-derived NO in limiting the magnitude of reperfusion injury in the postischemic heart (4) seems to be abolished in diabetes. This suggests that the presence of a functional iNOS gene (producing high levels of NO) during hyperglycemia might worsen myocardial damage during reperfusion injury, possibly through enhanced accumulation of peroxynitrite and nitrotyrosine.

One important finding of our study is the difference in myocardial infarct size between diabetic and nondiabetic mice. Infarct size is larger in both groups of STZ-induced diabetic mice compared with nondiabetic counterparts. Previous investigations of the extent of ischemic injury in diabetic myocardium have been controversial (15–18) despite overwhelming clinical evidence that the diabetic heart is highly sensitive to such injury (19). However, because the hyperglycemic STZ-injected mice are a model of type 1 diabetes, it is difficult to extrapolate conclusion to the clinical setting. One clinical finding relating to cardiovascular outcome during myocardial infarction consists of hyperglycemia, which is true for both types of diabetes and highly predictive of coronary death risk (19). Even if clinical studies that investigated the relationship

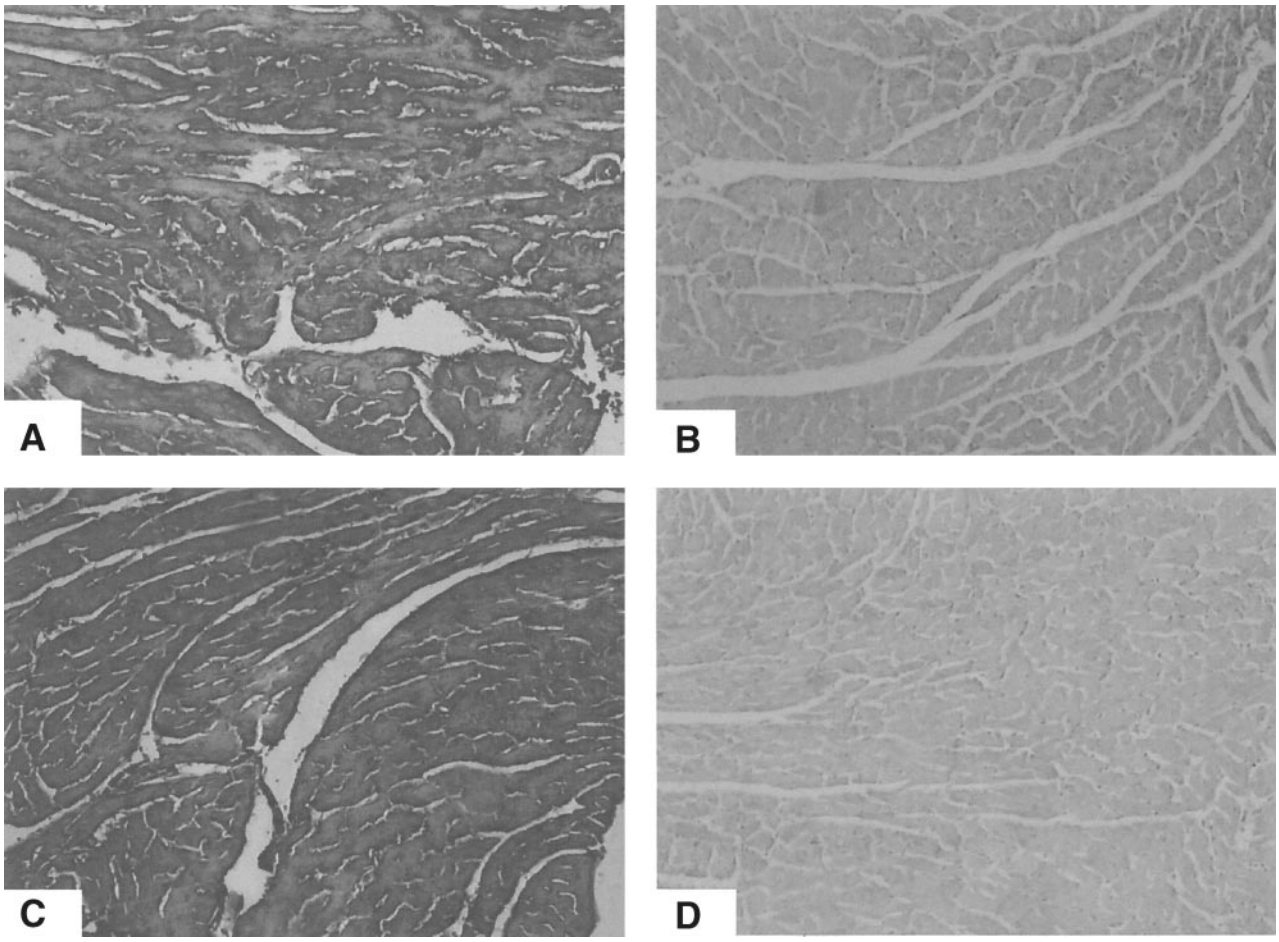

FIG. 5. Nitrotyrosine immunostaining in infarcted hearts from iNOS$^{+/+}$ non diabetic mice (*A*), iNOS$^{-/-}$ nondiabetic mice (*B*), iNOS$^{+/+}$ diabetic mice (*C*), and iNOS$^{-/-}$ diabetic mice (*D*). Significantly intense nitrotyrosine immunostaining was present in tissue from iNOS$^{+/+}$ nondiabetic hearts compared with iNOS$^{-/-}$ nondiabetic hearts ($P < 0.001$). nitrotyrosine immunostaining of hearts from iNOS$^{+/+}$ diabetic mice was significantly higher compared with both iNOS$^{+/+}$ nondiabetic ($P < 0.001$) and iNOS$^{-/-}$ diabetic mice ($P < 0.001$).

between hyperglycemia and mortality did not assess infarct size in their analysis, a critical role for glucose has recently been demonstrated by a linear relationship between blood glucose concentration and infarct size (20) and decreased survival and exaggerated left ventricular remodeling and failure (21) in diabetic or acutely hyperglycemic rats. The more extensive cardiac ischemic damage may be linked to a greater iNOS expression that may amplify the inflammatory process evoked by diabetes (22).

In recent years, it has been firmly established that inflammation not only contributes to the initiation and progression of atherosclerosis but is also a key player in the cardiac outcome of acute coronary syndromes (23). However, little is known about the potentially unique

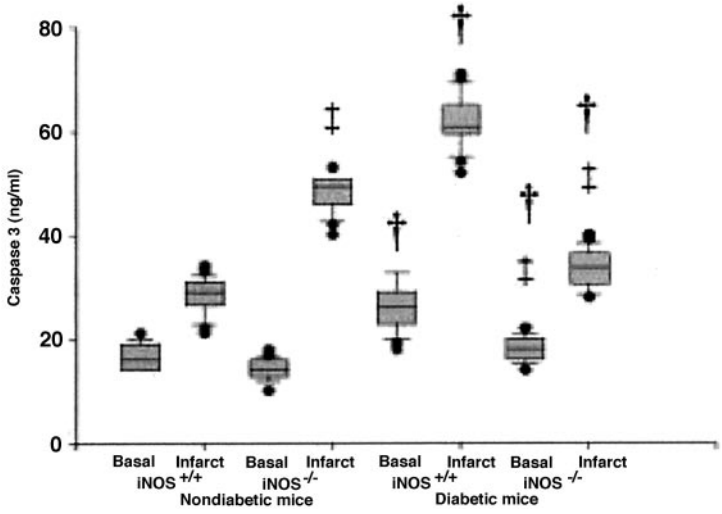

FIG. 6. Caspase-3 activity at baseline and after ischemia-reperfusion injury in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice with and without diabetes (Boxplot, a plot type that displays the 10th, 25th, 50th, 75th, and 90th percentiles as lines on a bar centered about the mean, and the 5th and 95th percentiles as error bars. The mean line and data points beyond the 5th and 95th percentiles can also be displayed). *$P < 0.01$ vs. iNOS$^{+/+}$ mice; ‡$P < 0.05$ vs. iNOS$^{+/+}$ mice; †$P < 0.001$ vs. nondiabetic mice.

features of this inflammatory process in diabetes. Several inflammatory markers have been associated with cardiovascular events, including cytokines and growth factors, which are released by activated macrophages (24). We found that, compared with nondiabetic mice, both iNOS$^{+/+}$ and iNOS$^{-/-}$ diabetic mice had higher tissue levels of cytokines implicated in plaque destabilization (IL-18) and future cardiovascular events (TNF-α, IL-6). Interesting enough, acute hyperglycemia in healthy subjects and in patients with impaired glucose tolerance increases the circulating levels of these cytokines (25), which are also able to stimulate the production of iNOS by mononuclear and mesenchymal cells (26). Hyperglycemia-induced oxidative stress (27), along with soluble advanced glycation end products and products of lipid peroxidation, possibly serves as a key activator of upstream kinases, leading to induction of inflammatory gene expression (28).

Our data also provide the evidence that after myocardial infarction, iNOS expression is associated with increased cytokine levels in the diabetic myocardium, as well as elevations of NO and nitrotyrosine levels, and apoptosis in the perinfarction area in wild-type compared with the iNOS$^{-/-}$ mutant mice. Furthermore, increases in NO production and nitrotyrosine levels in the wild-type diabetic mice are associated with increased myocardial injury, suggesting that increased NO production from iNOS enhances peroxynitrite formation, contributing to myocardial damage in diabetic mice after myocardial infarction as well as the increase of blood pressure. Against this background is the observation that peroxynitrite formed after myocardial infarction is not associated with increased myocardial injury in nondiabetic animals. As pharmacological studies (29) show that peroxynitrite-neutralizing agents are protective in nondiabetic animals, it is possible to hypothesize that in nondiabetic animals, iNOS-derived NO has multiple roles, such as maintenance of vascular tone and possibly inhibition of mononuclear cell recruitment (30). These all are beneficial roles and protect against myocardial ischemia-reperfusion injury. Some of iNOS-derived NO, however, ends up as peroxynitrite, which is deleterious. In nondiabetic iNOS-deficient animals, the protective effect of the basal NO produced is more important than the deleterious effect that is generated via peroxynitrite. Under elevated levels of superoxide in diabetes, more NO can react with superoxide, and therefore during myocardial ischemia-reperfusion injury more peroxynitrite is formed. This additional peroxynitrite exerts deleterious effects, which become more relevant than the protective effect of NO. Consistent with this hypothesis, iNOS deficiency brings back the level of injury exactly to the level of what is seen in the nondiabetic iNOS-deficient animals subjected to myocardial ischemia-reperfusion injury.

Recently it was demonstrated that in isolated rat hearts, acute exposure to high glucose increases iNOS gene expression, paralleled by a simultaneous increase of both NO and O_2^- production (3). The interaction of O_2^- with NO is very rapid and leads to inactivation of NO and production of the potent oxidant peroxynitrite (31). Because nitrotyrosine is considered a good marker of peroxynitrite formation (32), detection of high levels of nitrotyrosine in diabetic hearts of wild-type mice but not in diabetic and nondiabetic hearts of iNOS$^{-/-}$ mice is strongly suggestive of increased generation of peroxynitrite. The observation that the increased apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsies from patients with diabetes (33), as well as in hearts from STZ-induced diabetic rats (3), is selectively associated with levels of nitrotyrosine found in those cells supports our hypothesis. The mechanisms by which diabetes increases apoptotic myocardial cell death remain, at least in part, to be explained. The recent demonstration that cytokine-induced apoptosis is mediated by iNOS induction and peroxynitrite formation in primary cultures of neonatal rat myocytes (22) suggests a central role for proinflammatory cytokines in myocardial damage during ischemia, either acting directly on myocardial cells (34) or stimulating iNOS expression (35).

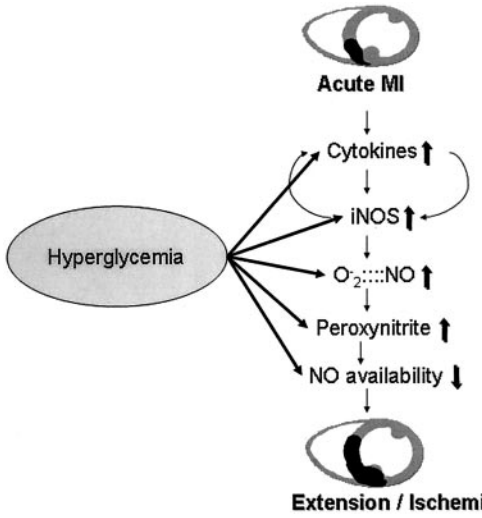

FIG. 7. Potential mechanism and mediators by which hyperglycemia may contribute to cardiac myocyte injury.

The present study introduces an additional aspect of how hyperglycemia might contribute to cardiovascular death in myocardial infarction patients: diabetic hyperglycemia results in increased tissue levels of proinflammatory cytokines, myocardial iNOS overexpression and NO production, and higher nitrotyrosine levels, leading to myocardial apoptosis and greater infarct size (Fig. 7). Moreover, although we cannot exclude that the systemic effects of iNOS absence might have influenced the response to ischemia, the divergent effect of normoglycemia and hyperglycemia on ischemic injury in iNOS$^{-/-}$ mice suggests that the level of blood glucose is more important than the systemic effect of iNOS deficiency in conditioning the ischemic response.

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