Reperfusion injury is not affected by blockade of P-selectin in the diabetic mouse heart

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Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H763–H769, 1999.—We examined the mechanisms responsible for myocardial ischemia-reperfusion (MI-R) injury in a well-characterized animal model of type II diabetes mellitus. Diabetic (db/db) mice and their littermate nondiabetic controls were subjected to 30 min of left anterior descending coronary artery occlusion and 2 h of reperfusion. Diabetic and nondiabetic mice experienced similar-sized areas at risk per left ventricle: 50.4 ± 2.0 and 53.4 ± 4.1%, respectively. However, myocardial necrosis (percentage of area at risk) was significantly greater (P < 0.001) in diabetic than in nondiabetic animals: 56.3 ± 2.8 and 27.2 ± 3.1%, respectively. Histological examination revealed significantly (P < 0.05) more neutrophils (PMNs) in the diabetic than in the nondiabetic hearts. Coronary endothelial expression of P-selectin was determined using radiolabeled monoclonal antibodies (MAbs). MI-R elicited a more intense (P < 0.05) upregulation of P-selectin in the ischemic zone of diabetic than of nondiabetic myocardium: 0.310 ± 0.034 and 0.161 ± 0.042 μg MAbs/g tissue. Immunoneutralization of P-selectin (RB40.34) reduced PMN accumulation in the diabetic myocardium but failed to reduce the extent of myocardial necrosis. Conversely, administration of an MAb directed against CD18 (GAME46) reduced PMN infiltration and attenuated the infarct size in the diabetic hearts. These results suggest that the diabetic heart is more susceptible to ischemia-reperfusion injury than normal myocardium. Furthermore, the mechanism of this injury may not be critically dependent on P-selectin in diabetic hearts.

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An established model (27) of murine MI-R was used to determine 1) whether MI-R injury differs between normal and diabetic mice, 2) the degree of PMN infiltration into the diabetic and nondiabetic hearts after ischemia-reperfusion, 3) the regional, in vivo expression of P-selectin in ischemic and nonischemic zones of normal and diabetic myocardium exposed to ischemia and reperfusion, and 4) the effect of P-selectin and CD18 immunoneutralization on the extent of myocardial necrosis in diabetic hearts subjected to ischemia and reperfusion.

**MATERIALS AND METHODS**

Transgenic mice. Our studies focused on the db/db diabetic mouse as an animal model of type II diabetes mellitus. Many studies (7, 11, 14, 32) support the validity of this mouse as a model of human diabetes mellitus. Briefly, the existence of studies (7, 11, 14, 32) support the validity of this mouse as a model of human diabetes mellitus. Briefly, the existence of diabetes in the db/db diabetic mouse is caused by a point mutation in the leptin receptor gene. This results in a variety of diabetes pathologies, including hyperglycemia, hypercholesterolemia, and obesity.

Male littermate C57BLKS/J -+/db (heterozygote control) and C57BLKSJ +/+db (homozygote diabetic) mice were purchased from Harlan and maintained on a normal rodent chow diet. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 86-23, revised 1985. Animal Resources Program, DRR/NIH, Bethesda, MD 20205), approved by the Council of the American Physiological Society, and with federal and state regulations. All experimental procedures were approved by the Louisiana State University Medical Center Animal Care and Use Committee.

Blood glucose and cholesterol determinations. Fifteen nondiabetic and 14 diabetic mice were anesthetized with pentobarbital sodium (100 mg/kg ip), and blood obtained was screened using a Sure Step glucose-monitoring system (Lifescan). In addition, 10 nondiabetic and 6 diabetic mice were also anesthetized and exsanguinated, and these blood samples were used for cholesterol determination. The serum was extracted from each sample and quantified for total cholesterol (Cholesterol 20, Sigma Diagnostics).

Arterial pressure and platelet counts. Nondiabetic (n = 7) and diabetic (n = 7) mice were anesthetized, instrumented with carotid artery catheters, and exsanguinated. The whole blood was placed in EDTA Microtainers (Becton-Dickinson). The samples were sent to LabCorp (BioVet Division, Research Triangle Park, NC) for the determination of circulating white blood cell and platelet counts.

Coronary P-selectin expression experiments. Radiolabeled P-selectin and isotype-matched control monoclonal antibodies (MAbs) were prepared as previously described (3). Two groups of mice were used in the P-selectin MAB study. The first group of nondiabetic (n = 8) and diabetic (n = 9) mice was instrumented with carotid artery cannulas. Monodonal radiolabeled (125I) antibody directed against P-selectin (RB40.34, Pharmingen) and a isotype-matched, nonbinding radiolabeled (131I) antibody (P-23, Pharmacia-Upjohn) were administered retrogradely into the carotid artery. After 5 min of circulation, a 50-µl plasma sample was drawn. The animal was then perfused with 15 ml of warm, heparinized bicarbonate-buffered saline, during exsanguination, to flush the excess P-selectin MAB and nonbinding control antibody. The heart was excised and weighed, and cardiac radioactivity was measured using an automatic gamma-counter (1480 Wizard, Wallac) to determine constitutive P-selectin expression. Additionally, the following items were also measured for γ-radiation to correct for unbound antibodies: the 50-µl plasma sample, syringe, intravenous catheter, and 2 µl of the original antibody mixture. The gamma-counts of these items were factored into the determination of P-selectin expression with use of the following equation: (125I cpm/g - 131I cpm injected) ÷ (131I cpm/g - 125I cpm injected).

In the second group, nondiabetic (n = 7) and diabetic (n = 6) mice were subjected to 30 min of left anterior descending coronary artery (LAD) ischemia. The occluder was released, and the coronary arteries were allowed to reperfuse for 20 min. At 15 min of reperfusion, the mice were injected with the radiolabeled P-selectin and the radiolabeled isotype-matched control MAbs. After 5 min, a 50-µl plasma sample was drawn from the carotid artery cannula. The animal was then flushed with 15 ml of warm, heparinized bicarbonate-buffered saline through the jugular vein catheter and exsanguinated via the carotid catheter. LAD reperfusion was followed by infusion of Evans blue to delineate the ischemic zone from the nonischemic zone. The heart was excised and serially sectioned. The ischemic and nonischemic zones were separated, weighed, and measured for radioisotopic activity.

Surgical procedures. Animals were anesthetized with pentobarbital sodium (100 mg/kg ip). Anesthesia was maintained via supplemental doses of pentobarbital sodium (30 mg/kg ip) and meperidine (10 mg/kg ip) as needed. Mice were secured to the operating table by taping the extremities. A 4-0 silk ligature was placed behind the upper incisors and pulled tautly to extend the neck. To confirm normohyperglycemic states, a preoperative blood sample was obtained from the tail vein and screened using a Sure Step glucose-monitoring system. A midline incision was made from the xiphoid process to the submentum. The salivary glands were separated from the midline to allow access to the trachea. A tracheotomy was then performed to facilitate breathing. A section of PE-90 tubing was inserted into the animal’s trachea and connected via a loose junction to a respirator (model 683 rodent respirator, Harvard Apparatus). The respirator’s tidal volume was set at 1.2 ml/min, and the rate was set at 120 strokes/min; supplementation was with 100% oxygen. The right carotid artery was then cannulated with PE-10 tubing to monitor mean arterial pressure and heart rate. The arterial cannula was connected to a blood pressure transducer and a blood pressure monitor (model BP-1, World Precision Instruments). This monitor was then interfaced with a MacLab/8e (AD Instruments) with real-time monitoring on a PowerMac 8500/150 (Apple Computers).

After an equilibration period of 10 min, a thoracotomy was performed. With use of an electrocautery (model 100, Geiger Instrument), an incision was made to the left of the sternum. The pericardial sac was then removed. Ligation of the LAD was performed using a 7-0 silk suture attached to a BV-1 needle (Ethicon). A small piece of polyethylene tubing was used to secure the ligature without damaging the artery. The chest wall was approximated and covered with waxed paper (Parafilm) to prevent desiccation. All animals in the infarct size determination protocol were subjected to 30 min of LAD occlusion and 120 min of reflow.

Determination of area at risk and infarct size. At the conclusion of the 2-h period of reperfusion, the LAD was religated with 7-0 silk suture. Evans blue (1.2 ml, 1.0% Sigma Chemical) was retrogradely injected into the carotid artery catheter to delineate the in vivo area at risk.

At the end of the protocol the heart was excised and fixed in a 1.5% solution of Seaplaque agarose gel (FMC BioProducts). After the gel solidified, a Mliwain tissue chopper (Brinkmann Instruments) was used to section the heart perpendicularly to the long axis in 1-mm portions. The 1-mm sections were placed in individual wells of a six-well cell culture plate, with
the basal side exposed. Each slice was then counterstained with 3.0 ml of 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical) solution for 5 min at 37°C. Each slice was weighed and visualized under a dissecting microscope (model SZ40, Olympus America) equipped with a charge-coupled device iris-color video camera (Sony Electronics). The left ventricular area, area at risk, and area of infarction for each slice were then determined by computer planimetry with use of NIH Image (version 1.57) software. The size of the myocardial infarction was determined by the following previously described (26) equation: weight of infarction = (A_1 \times W_{t1}) + (A_2 \times W_{t2}) + (A_3 \times W_{t3}) + (A_4 \times W_{t4}) + (A_5 \times W_{t5}), where A is percent area of infarction by planimetry from subscripted numbers 1–5 representing sections and Wt is the weight of the same-numbered sections.

Myocardial histology. Routine histological staining was performed on multiple midventricular cardiac sections to determine the extent of PMN infiltration into the ischemic-reperfused myocardium. Nondiabetic (n = 3) and diabetic (n = 3) hearts were subjected to 30 min of myocardial ischemia and 120 min of reperfusion and stained as described above. In additional MI-R studies, diabetic mice were treated with P-selectin MAb (RB40.34) or a CD18 MAb (GAME46), as described above. Five of the RB40.34- and five of the GAME46-treated hearts were included for histological analysis of PMN accumulation. The hearts were stored overnight in 4.0% paraformaldehyde at 4°C. The tissue was cut into sections and dehydrated using graded acetone washes at 4°C. Tissue sections were embedded in plastic (ImmunoBed, Polysciences), and 4-μm-thick sections were cut and transferred to Vecatabond-coated slides (Vector Laboratories). The slides were soaked in 95% ethanol for 10 min to remove some of the plastic embedding and to allow the tissue to stain. After the 10-min ethanol wash, the tissue sections were stained with hematoxylin solution (Gill no. 3, Sigma Chemical) for 10 min or Giemsa stain (Sigma Chemical) for 3 min. The slides were then observed microscopically, and the number of PMNs were counted per field of view. For each of the 3 hearts examined the number of PMNs was counted in 4 fields for a total of 12 fields.

Administration of P-selectin and CD18 MAb. The animals were surgically prepared as described above. Before ischemia, the mice received an intravenous injection (150 μl total volume) of 1 mg/kg anti-mouse P-selectin MAb (RB40.34; n = 6 mice) or 1 mg/kg murine-specific CD18 MAb (GAME46, Endogen; n = 7 mice). All drugs were dissolved in 0.9% normal saline. The heart rates and mean arterial blood pressures were monitored throughout the experiments.

Statistical analyses. The infarct sizes and hemodynamic data were analyzed by ANOVA with Scheffé’s post hoc test. Preoperative blood glucose and serum cholesterol levels. Preoperative, nonfasting, whole blood samples were obtained from nondiabetic (n = 15) and diabetic (n = 14) mice to confirm their normoglycemic and hyperglycemic states (Fig. 1). Mean blood glucose level was 115.6 ± 5.5 and 468.5 ± 16.7 mg/dl in nondiabetic and diabetic mice, respectively (P < 0.001 vs. nondiabetic). In addition, diabetic mice (n = 6) exhibited significantly (P < 0.001) elevated total serum cholesterol levels (Fig. 2) of 144.3 ± 10.0 mg/dl, while the nondiabetic mice (n = 10) had total serum cholesterol levels of 51.5 ± 2.8 mg/dl.

Peripheral PMN and platelet counts. Data for PMN and platelet counts in whole blood from nondiabetic (n = 7) and diabetic animals (n = 7) are summarized in Fig. 3. There was no significant difference in PMN counts between nondiabetic and diabetic mice: 0.50 ± 0.06 and 0.71 ± 0.09 × 10^3 cells/µl whole blood, respectively. Similarly, no difference was observed in platelet counts between nondiabetic and diabetic mice: 10.1 ± 0.5 and 10.6 ± 0.2 × 10^3 cells/µl whole blood, respectively.

Constitutive P-selectin expression. A dual-radiolabeled MAb technique was used to determine the in vivo constitutive endothelial cell expression of P-selectin in nondiabetic (n = 8) and diabetic (n = 9) hearts. There was no significant difference in constitutive myocardial P-selectin expression (Fig. 4) between diabetic and nondiabetic mice.
nondiabetic mice: 0.006 ± 0.001 and 0.006 ± 0.002 µg MAb/g tissue, respectively.

MI-R-induced P-selectin expression. P-selectin was also determined in nondiabetic (n = 7) and diabetic (n = 6) mouse hearts after 30 min of coronary occlusion and 20 min of reperfusion (Fig. 5). P-selectin expression in the nonischemic zone was similar in the nondiabetic and diabetic hearts: 0.033 ± 0.011 and 0.037 ± 0.011 µg MAb/g tissue, respectively. However, P-selectin was significantly increased in the ischemic zone of nondiabetic (P < 0.05) and diabetic hearts (P < 0.001) compared with their respective nonischemic zones. P-selectin expression was 0.161 ± 0.042 and 0.310 ± 0.034 µg MAb/g tissue in the nondiabetic and diabetic ischemic zones, respectively (P < 0.05 vs. nondiabetic).

Hemodynamic data. Blood pressures and heart rates were recorded for nondiabetic (n = 15) and diabetic (n = 14) animals throughout the myocardial ischemia experiments and reported in Table 1. In addition, the rate-pressure product was calculated to provide an index of oxygen demand. No significant group differences in mean arterial blood pressure, heart rate, or rate-pressure product were observed at any time point in the experiments.

Exclusion criteria. A total of 17 nondiabetic and 24 diabetic mice entered the MI-R infarct size determination protocol. Two nondiabetic and 10 diabetic animals

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Values are means ± SE. HR, heart rate; MABP, mean arterial blood pressure; RPP, rate-pressure product ([HR × MABP]/1,000).
died during MI-R. Seven diabetic mice given GAME 46 entered the protocol and all seven were included in the analysis. Of the seven mice given RB 40.34, six survived to be included in the infarct assessment data. Statistical analysis of survival data revealed that survival was not statistically significant between the nondiabetic and diabetic groups (P = 0.07).

Myocardial PMN accumulation. Graphic interpretation of PMN counts within the ischemic-reperfused myocardium after 30 min of myocardial ischemia and 120 min of reperfusion are presented in Fig. 6. The nondiabetic hearts contained significantly fewer (P < 0.01) PMNs than the diabetic hearts: 18.8 ± 2.6 vs. 52.9 ± 4.5 PMNs/field.

Myocardial area at risk and infarct size. Representative photomicrographs of area at risk and infarct staining are presented in Fig. 7. Despite similar-sized areas at risk, the diabetic hearts suffered from a significantly larger area of infarction after MI-R. In general, the untreated diabetic hearts suffered infarcts that were nearly transmural in appearance. Conversely, the nondiabetic littermates presented infarcts characterized by mid to epicardial necrosis. Data for area at risk and infarct size are summarized in Fig. 8. Both groups of animals experienced similar-sized ischemic zones per left ventricle: 50.4 ± 2.0 and 53.4 ± 4.1% of left ventricle at risk in diabetic and nondiabetic animals, respectively (not significant). Infarct size was 27.2 ± 3.1 and 57.1 ± 2.5% of the area at risk in nondiabetic and diabetic mice, respectively (P < 0.01).

Data from experiments in which diabetic mice were given P-selectin MAb (n = 6) and CD18 MAb (n = 7) are also shown in Fig. 8. Both experimental groups had similar-sized areas at risk per left ventricle: 52.1 ± 3.6 and 51.7 ± 1.7% for RB40.34 and GAME46, respectively. However, only GAME46 significantly reduced (P < 0.05) myocardial necrosis in the ischemic zone (ratio of infarct size to area at risk) of diabetic animals: 47.2 ± 9.4 and 34.4 ± 8.1% for RB40.34 and GAME46, respectively.

**DISCUSSION**

The present study clearly demonstrates that the diabetic myocardium exhibits an exaggerated response to acute MI-R. Furthermore, this injury appears to be related to enhanced PMN infiltration through a CD18-dependent mechanism. Data obtained from diabetic mouse hearts treated with a P-selectin-neutralizing MAb suggest that PMN-mediated myocardial reperfusion injury is not critically dependent on P-selectin function. Our results support clinical observations of human diabetics suffering more severe heart attacks than their nondiabetic peers (29, 36). Consequently, this investigation focused on the possible injurious mechanism(s) that may be hyperactive in this thoroughly characterized animal model of diabetes mellitus. Although previous investigations have shown other models of animal diabetes to be cardioprotective (4, 21), the present study is the first investigation into MI-R injury in the db/db mouse.

Although human diabetes mellitus is a manifold disease, the db/db mouse model of type II diabetes mellitus mimics several pathologies commonly associated with human diabetes, the foremost of which is their profound hyperglycemia. Blood glucose in db/db mice cannot be controlled by exogenous insulin because of a progressive insensitivity to insulin (15). In addition, we demonstrated that db/db mice exhibit hypercholesterolemia when fed a normal rodent chow diet. Furthermore, several investigators have described histopathological changes in various organs, including pancreatitis (20), nephropathy (1, 23), neuropathy (20), and cardiomyopathy (7). Although the db/db mouse is not a perfect model of the (various) manifestations of human diabetes mellitus, these regional pathological changes are consistent with the findings of the present study.
states confirm clinical observations in humans and support the validity of this murine model as a vehicle for studying the pathophysiology of type II diabetes mellitus.

In nondiabetic animals, P-selectin is necessary for the sequestration of PMNs and their ensuing tissue damage (17). In previous studies (18, 19), our laboratory has shown that inhibition of P-selectin expression reduces myocardial necrosis after MI-R in nondiabetic animals. Others have reported increased circulating forms of various leukocyte-endothelial cell adhesion molecules (16) as well as increased tissue expression of cell adhesion molecules in diabetic animals (30) and patients (25). Furthermore, leukocytes isolated from diabetics produce more toxic oxygen radicals (6, 35) and have been shown to contribute to capillary nonperfusion in the diabetic retina (31). One of the most striking findings in this study is the pronounced expression of P-selectin in the coronary vasculature of diabetic mice after MI-R. It is important to note that this difference was not caused by constitutive changes in P-selectin expression in the coronary vasculature. However, administration of an MAb specifically directed against murine P-selectin did not significantly diminish infarct size. Administration of the P-selectin MAb did significantly abrogate PMN sequestration in the diabetic myocardium subjected to ischemia and reperfusion. Therefore, this profound expression of P-selectin within the coronary circulation of the diabetic heart may play an ancillary role in the development of myocardial infarction.

Although anti-P-selectin therapy did not attenuate infarct size, PMNs were apparently involved in the development of myocardial necrosis. The presence of twofold more PMNs in the diabetic myocardium subjected to ischemia-reperfusion, coupled with the efficacy of anti-CD18 therapy, strongly suggests a prominent role for PMNs in diabetic MI-R injury. The inability of P-selectin blockade to reduce infarct size raises several important possibilities for the mechanism of injury in the diabetic hearts. First, the shear rates in the diabetic heart subjected to ischemia-reperfusion may be low enough to allow P-selectin-independent PMN rolling. A previous study (24) has demonstrated lower shear rates in diabetic than in nondiabetic hearts. There also exists the possibility of constitutive expression of E-selectin in the diabetic hearts. Either of these alternatives might explain the ineffectiveness of P-selectin blockade in this model.

Hypercholesterolemia may be another factor that could effect a more profound inflammatory response. Others have reported (8, 10, 33) that hypercholesterolemia exacerbates ischemia-reperfusion injury via enhanced PMN-endothelial cell interactions. It has previously been demonstrated (10) that chronic hypercholesterolemia results in enhanced leukocyte adhesion and inflammation in the microcirculation of low-density lipoprotein receptor-deficient mice after administration of a number of inflammatory stimuli. In addition, it has also been reported (8, 33) that MI-R injury is aggravated in hypercholesterolemic rabbits. It is possible that the elevated circulating cholesterol levels in the db/db mice may have contributed to the greater extent of myocardial reperfusion injury. However, it is difficult to delineate the precise contributions of hypercholesterolemia and diabetes to postischemic myocardial injury in our experimental model system.

In summary, the present study demonstrates exacerbated myocardial infarction after ischemia and reperfusion in a genetic animal model of type II diabetes mellitus. The enhanced myocardial injury cannot be attributed to altered hemodynamic conditions in the diabetic heart, elevated circulating PMN counts, or increased constitutive expression of P-selectin. Although P-selectin expression was elevated early during

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**Fig. 7.** Area at risk per left ventricle (AAR/LV) and infarcts per area at risk (Inf/AAR). There were no significant group differences in AAR/LV. Inf/AAR was significantly larger (**P < 0.01**) in diabetic (n = 14) than in nondiabetic hearts (n = 15). Only anti-CD18 therapy (GAME46) significantly (**P < 0.05**) reduced infarct size in diabetic hearts (n = 7). MAb (RB40.34) directed against P-selectin did not alter myocardial necrosis (n = 6).

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**Fig. 8.** Myocardial neutrophil (PMN) accumulation after 30 min of myocardial ischemia and 120 min of reperfusion in nondiabetic (n = 3) and diabetic (n = 3) mouse hearts and in diabetic (Db) hearts treated with P-selectin MAb RB40.34 (n = 5) or anti-CD18 MAb GAME46 (n = 5). A total of 12 fields were examined in each heart, and number of PMNs per field was determined. Myocardial PMN accumulation was significantly (**P < 0.01**) greater in diabetic than in nondiabetic hearts, and this enhanced PMN influx was significantly attenuated after treatment with RB40.34 or GAME46. *P < 0.05 vs. GAME46.
reperfusion, P-selectin immunoblockade did not reduce myocardial infarct size in the diabetic heart. However, immunoneutralization of CD18 reduced the infarct size in the diabetic heart by ~40%. Consequently, a significant portion of the diabetic M1-R injury appears to be a result of an enhanced inflammatory response mediated by PMNs. Accordingly, these novel findings may serve as a nidus for future animal investigations into the molecular mechanisms of diabetic M1-R injury.

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