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Altered Susceptibility to Ischemia-Reperfusion Injury in Isolated-Perfused Hearts of Short-Term Diabetic Rats Associated With Changes in Non-enzymatic Antioxidants

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ABSTRACT—The effects of short-term (2-week) diabetes on myocardial ischemia-reperfusion (I-R) injury and associated changes in myocardial non-enzymatic antioxidant level were examined. Isolated-perfused hearts prepared from control and diabetic rats were subjected to increasing periods of ischemia and reperfusion, and myocardial I-R injury was assessed by measuring the extent of lactate dehydrogenase (LDH) leakage and contractile force recovery. While a brief period (20 min) of post-ischemic reperfusion caused a smaller extent of LDH leakage, the prolonged period (40 min) of reperfusion produced a greater degree of I-R injury in diabetic hearts, as indicated by the impaired recovery of contractile force. The apparent protection against I-R injury in diabetic hearts during the early phase of post-ischemic reperfusion was associated with increases in myocardial reduced glutathione/ascorbic acid and α-tocopherol levels, with the effect on α-tocopherol being most prominent. Insulin treatment could reverse the diabetes-associated changes in susceptibility to myocardial I-R injury and antioxidant response. The ensemble of results indicates that the myocardium isolated from short-term diabetic rat can produce a beneficial antioxidant response to I-R challenge, which may, in turn, be attributable to the decreased susceptibility to I-R injury observable during the early phase of reperfusion.

Keywords: Diabetes, Myocardial ischemia-reperfusion injury, Glutathione, Ascorbic acid, α-Tocopherol

Ischemic heart disease is the major cause of morbidity and mortality in patients suffering from diabetes (1, 2). More than one half of all diabetic patients were found to have died of sequelae arising from myocardial ischemia such as acute myocardial infarction and heart failure (3, 4). While the presence of diabetic complications such as autonomic dysfunction (5, 6) and microvascular dysfunction (7, 8) can increase the risk of cardiac death, the enhanced susceptibility of the myocardium to ischemia-reperfusion (I-R) damage under the diabetic condition may also play a role in causing the poor cardiac prognosis for diabetic patients (3, 4). Diabetic patients were found to have larger infarction (9) and high mortality rate following an acute episode of myocardial ischemia (10). Experimental studies have also shown an enhanced vulnerability of diabetic rat hearts to ischemic injury (11, 12). On the other hand, there were also studies showing that the diabetic heart was more resistant to ischemic injury (13, 14). The inconsistent findings may result from the varying ischemic and/or reperfusion conditions in these experimental studies. Given the involvement of oxy-radical-mediated processes in the pathogenesis of myocardial I-R injury (15) and diabetic complications (16), the increased susceptibility of diabetic hearts to I-R injury may be related to the functional impairment in the myocardial antioxidant defense system. In this regard, it has, however, been reported that acute diabetes could cause considerable increases in myocardial catalase and superoxide dismutase activities even though myocardial malondialdehyde level was increased (17). In addition, the decreased myocardial glutathione level as well as catalase and superoxide dismutase activities were found to be associated with a lower sensitivity of short-term diabetic rat hearts to experimentally induced myocardial infarction (18). The apparent inconsistency in diabetes-induced alterations in the myocardial antioxidant system and susceptibility to oxidative tissue damage may probably reflect a complex interaction between the diabetic state and myocardial antioxidant system in response to the increased oxidative stress conditions. In order to investigate the possible

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alteration in myocardial antioxidant response in diabetic hearts, we examined the myocardial susceptibility to I-R injury in parallel with the I-R-induced changes in non-enzymatic antioxidant levels in isolated-perfused hearts prepared from short-term (2-week) diabetic rats that were subjected to increasing periods of ischemia and reperfusion.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ), reduced glutathione (GSH), ascorbic acid (V_C) and α-tocopherol (V_E) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography were of HPLC grade.

Induction of diabetes and animal care

Diabetes was induced in 8 – 10-week-old female Sprague Dawley rats by a single intraperitoneal injection of STZ (prepared in ice-cold 0.1 M citrate buffer, pH 4.5) at a dose of 45 mg/kg. A previous study from our laboratory also used female rats to investigate the effect of herbal extract pretreatment on myocardial I-R injury (19). Non-diabetic animals (control) were injected with the buffer only (1 ml /kg body weight). The diabetic state was confirmed by the presence of glucosuria as determined by using glucose test strips (Glukotest; Boehringer, Mannheim, Germany) on the second day after STZ injection. Observation from a previous study indicated that the blood glucose level was found to be 1.6-fold higher in 2-week STZ-induced diabetic rats than that of the non-diabetic control (20). All animals were housed in an air/humidity-controlled room at the Animal Care Facilities of the Hong Kong University of Science and Technology, with a 12-h dark/light cycle, and allowed food and water ad libitum. Control and diabetic rats were assigned to various groups and I-R experiments were performed 2 weeks after the induction of diabetes.

Insulin treatment

The day of STZ injection was assigned as day 0. Insulin treatment began on day 3 and continued until the night before sacrifice (day 13). Diabetic animals with insulin treatment received twice daily intramuscular injection of insulin (2 U Actrapid MC + 2 U Monotard MC at 1100 h and 3 U Actrapid MC + 3 U Monotard MC at 2100 h). The same insulin treatment regimen was found to be able to decrease blood glucose well below the non-diabetic level in 2-week diabetic rats (20). I-R experiments were performed on day 14.

Isolated-perfused rat heart

The heart from diethyl ether-anesthetized rat was excised quickly and immediately immersed in ice-cold and heparinized (50 unit/ml) saline. The aorta was cannulated and then transferred to the warm and moist chamber of a perfusion apparatus. The heart was retrogradely perfused according to the Langendorff method as described in ref. 21. The apex of the heart was attached via a metal hook to an unextendable cotton thread that was connected to a force displacement transducer (Model FT03; Grass, Quincy, MA, USA), and the isometric contractions of the heart were recorded on a polygraph (Model 7-8P, Grass).

Myocardial I-R

After an initial 30-min of perfusion for equilibration, the isolated heart was subjected to a 20- or 40-min period of 'no-flow' normothermic global ischemia followed by a 20- or 40-min reperfusion. The non-ischemic control (non-I-R) hearts from control or diabetic animals were perfused for 60 or 80 min after equilibration, without subjecting them to ischemia. Coronary effluent was collected in 1-min fractions at increasing time intervals during the course of equilibration and reperfusion. Each fraction was immediately put on ice until assay. Myocardial I-R injury was assessed by measuring the extent of lactate dehydrogenase (LDH) leakage during the reperfusion period. LDH activity in all fractions of coronary effluent was assayed as described in ref. 21. The extent of LDH leakage was estimated by computing the area under the curve of the graph plotting the percent LDH activity (with respect to the mean preischemic value measured during the equilibration period) against the reperfusion time (1 – 20 or 40 min) as described in ref. 21.

Biochemical analysis

Myocardial tissue samples were rinsed with ice-cold homogenizing buffer (50 mM Tris(hydroxymethyl) aminomethane, 0.1 mM EDTA, pH 7.6) after the I-R experiment. Tissue homogenate was prepared by homogenizing 0.8 g of myocardial tissue in 8 ml ice-cold homogenizing buffer with two 10-s bursts of a tissue disintegrator (Ultra Turrax T25; Ika, Staufen, Germany) at 13,500 rpm, and the sample was analyzed for non-enzymatic antioxidants. Myocardial GSH level was measured by an HPLC method modified from Reed et al. (22) as described in ref. 23. Myocardial V_C and V_E levels were also measured by HPLC methods modified from Liau et al. (24) and Sadrzadeh et al. (25), respectively, as described in ref. 23.

Statistical analyses

Data were analyzed by one-way ANOVA followed by Duncan's multiple range test to detect the inter-group difference. Significant difference was determined when $P<0.05$.

RESULTS

I-R-induced myocardial damage in control and diabetic hearts

I-R caused tissue damage in isolated perfused rat hearts, as indicated by the increase in the extent of LDH leakage (Fig. 1a). While the extension of the ischemic period from 20 to 40 min could increase the extent of I-R injury, the prolonged period of reperfusion (i.e., 40 min) only caused additional tissue damage in hearts subjected to 40 min of ischemia. The diabetic state did not alter the extent of LDH leakage in perfused (non-I-R) hearts (data not shown), but it significantly reduced the extent of LDH leakage by 72% and 75%, respectively, at 20 min of reperfusion following 20- and 40-min of ischemia in I-R hearts (Fig. 1a). However, the extent of LDH leakage in diabetic hearts was significantly increased following 40-min post-ischemic reperfusion, with values being not significantly different from those of the non-diabetic I-R control.

Myocardial I-R damage was associated with the impairment in contractile force, as indicated by the decrease in the extent of contractile force recovery after post-ischemic reperfusion (Fig. 1b). Prolonged reperfusion (i.e., 40 min) following 20-min of ischemia caused notable recovery of contractile force, but this was not observable in hearts subjected to 40-min of ischemia. On the other hand, the protracted reperfusion time course of diabetic hearts caused a progressive deterioration of contractile force, particularly following 40-min of ischemia, with the extent of contractile force recovery being significantly smaller (50%) than that of the non-diabetic I-R control (Fig. 1b).

I-R-induced changes in myocardial non-enzymatic antioxidants in control and diabetic hearts

Under the present experimental conditions, I-R did not produce any detectable changes in myocardial GSH level except under the most severe condition (40-I, 40-R), in which the GSH level was significantly reduced by 13%

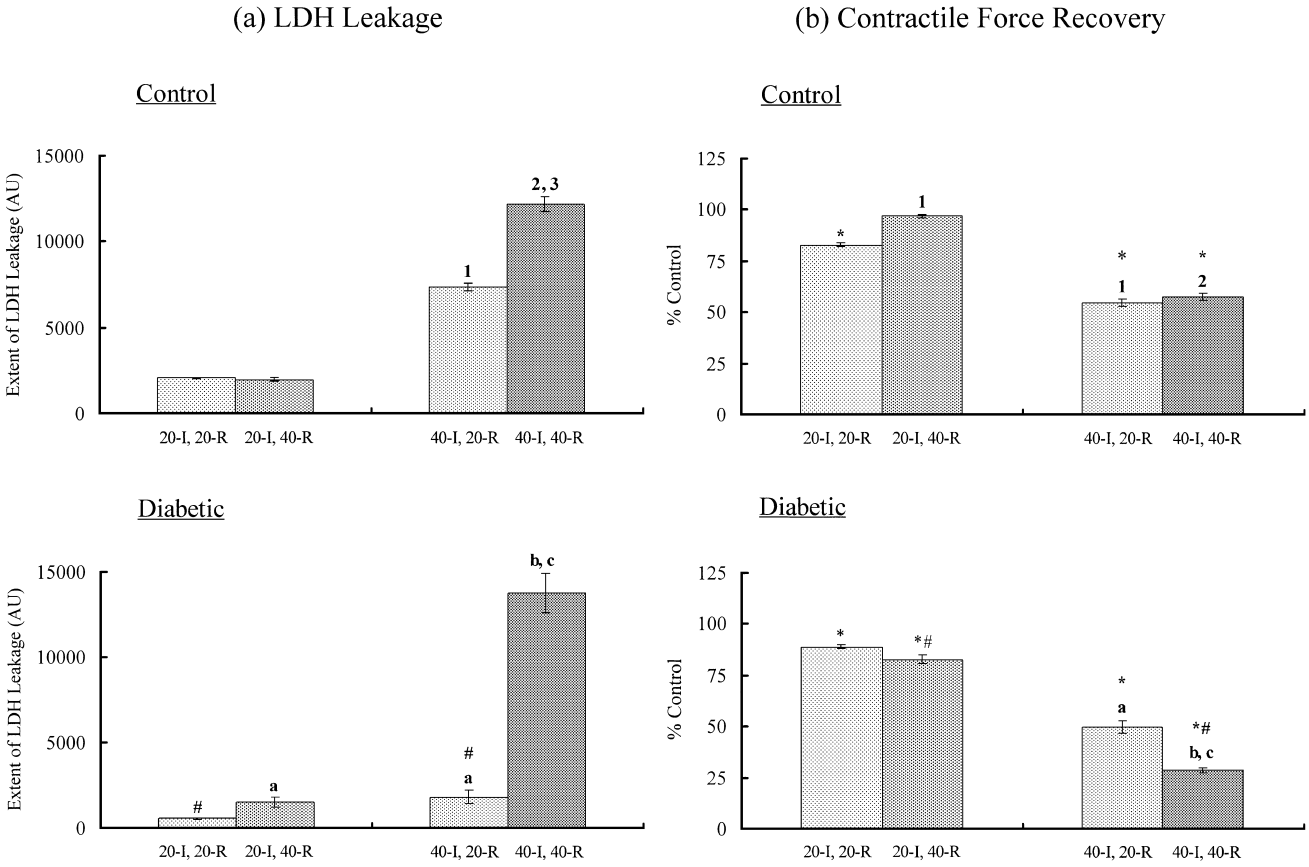


Fig. 1. Ischemia-reperfusion (I-R) injury in isolated-perfused control and diabetic rat hearts. Diabetes was induced by a single intraperitoneal injection of STZ at a dose of 45 mg/kg. Isolated hearts prepared from control and diabetic rats were subjected to increasing periods of ischemia (I) and reperfusion (R). Myocardial I-R injury was assessed by measuring the extent of LDH leakage (a) and contractile force recovery (b) as described in Materials and Methods. The extent of LDH leakage from non-I-R (i.e., perfused) hearts was subtracted from the respective I-R values. Each bar is the mean ± S.E.M., with n = 5. Significantly different from [1]20-I, 20-R control group; [2]20-I, 40-R control group; [3]40-I, 20-R control group; [a]20-I, 20-R diabetic group; [b]20-I, 40-R diabetic group; [c]40-I, 20-R diabetic group; *respective non-I-R group; and [#]respective I-R control group; $P<0.05$.

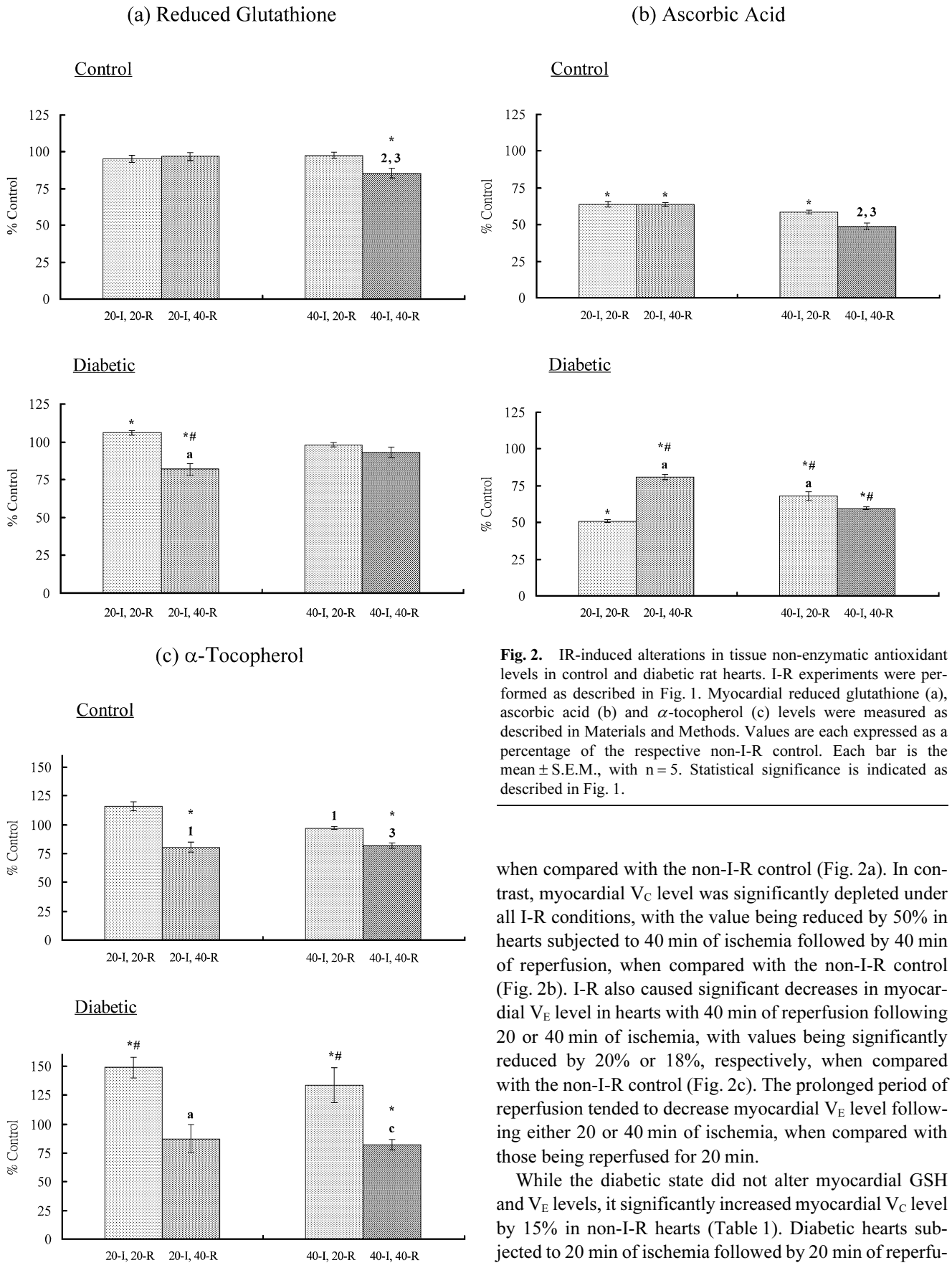


Fig. 2. IR-induced alterations in tissue non-enzymatic antioxidant levels in control and diabetic rat hearts. I-R experiments were performed as described in Fig. 1. Myocardial reduced glutathione (a), ascorbic acid (b) and α-tocopherol (c) levels were measured as described in Materials and Methods. Values are each expressed as a percentage of the respective non-I-R control. Each bar is the mean ± S.E.M., with n = 5. Statistical significance is indicated as described in Fig. 1.

when compared with the non-I-R control (Fig. 2a). In contrast, myocardial V_C level was significantly depleted under all I-R conditions, with the value being reduced by 50% in hearts subjected to 40 min of ischemia followed by 40 min of reperfusion, when compared with the non-I-R control (Fig. 2b). I-R also caused significant decreases in myocardial V_E level in hearts with 40 min of reperfusion following 20 or 40 min of ischemia, with values being significantly reduced by 20% or 18%, respectively, when compared with the non-I-R control (Fig. 2c). The prolonged period of reperfusion tended to decrease myocardial V_E level following either 20 or 40 min of ischemia, when compared with those being reperfused for 20 min.

While the diabetic state did not alter myocardial GSH and V_E levels, it significantly increased myocardial V_C level by 15% in non-I-R hearts (Table 1). Diabetic hearts subjected to 20 min of ischemia followed by 20 min of reperfu-

Table 1. Non-enzymatic antioxidant levels in isolated-perfused control and diabetic rat hearts

	GSH (nmol/mg tissue)	V_C (μg/g tissue)	V_E (μg/g tissue)
Control	1.04 ± 0.04	69.5 ± 3.2	19.4 ± 1.3
Diabetic	1.01 ± 0.04	79.7 ± 2.7[#]	20.3 ± 2.2

Isolated-perfused hearts were prepared from control and diabetic rats. After the equilibration, isolated hearts were perfused for 60 or 80 min. Myocardial reduced glutathione (GSH), ascorbic acid (V_C) and α-tocopherol (V_E) levels were analyzed as described in Materials and Methods. Since there were no detectable differences between values obtained from the 60-min and 80-min perfused (non-I-R) control or diabetic hearts, data from both groups were pooled. The value given is the mean ± S.E.M., with n = 10 in each group. [#]Significantly different from the control group, $P<0.05$.

sion showed a slight (6%) but significant increase in tissue GSH level, whereas a 40 min of post-ischemic reperfusion significantly reduced myocardial GSH level by 17%, when compared with the diabetic non-I-R control (Fig. 2a). No detectable differences in tissue GSH level were observed in diabetic hearts subjected to 40 min of ischemia followed by 20 or 40 min of reperfusion. Although myocardial V_C level was also significantly depleted in diabetic hearts under all I-R conditions, tissue levels of V_C in ischemic-reperfused diabetic hearts (except that of 20-I, 20-R) were significantly higher than the respective non-diabetic I-R control (Fig. 2b). Notably, the prolonged period of reperfusion after 20 min of ischemia caused a 30% increase in myocardial V_C level in diabetic hearts, when compared with that being reperfused for 20 min (Fig. 2b). Reperfusing diabetic hearts after 20 or 40 min of ischemia for 20 min produced a dramatic increase in tissue V_E level, with values being 49% or 34%, respectively, higher than the non-I-R diabetic control (Fig. 2c). The prolonged reperfusion caused a huge depletion in myocardial V_E level, with values being reduced to the level similar to those of non-diabetic I-R control.

Effect of insulin pretreatment on I-R-induced injury in diabetic hearts

An I-R condition of 20 min ischemia followed by 20 min of reperfusion was adopted to examine the effect of insulin treatment on I-R injury in diabetic hearts. As shown in Table 2, insulin treatment resulted in a significant increase in the extent of LDH leakage by onefold, when compared with the unpretreated diabetic I-R control. However, insulin treatment did not change the extent of contractile force recovery in the ischemic-reperfused diabetic hearts. The reversal effect of insulin treatment was associated with a significant increase in myocardial V_C level (17%) and decrease in myocardial V_E level (33%), when compared with the unpretreated diabetic control. However, myocardial GSH level remained relatively unchanged.

DISCUSSION

The effects of short-term (2-week) diabetes on myocardial I-R injury and the I-R-induced changes in myocardial non-enzymatic antioxidant levels were examined in isolated-perfused rat hearts. The susceptibility of diabetic hearts to I-R injury seemed to be dependent on the reperfusion time course. While a brief period of post-ischemic reperfusion caused a smaller extent of LDH leakage, the prolonged period of reperfusion produced a greater extent of I-R injury in diabetic hearts, as indicated by the impaired recovery of contractile force. The increased extent of myocardial I-R injury, particularly in the case of diabetic heart, produced by the prolonged period of post-ischemic reperfusion may be attributed to the prevailing oxy-radical-mediated reactions occurring during reperfusion (26). Results obtained from other studies regarding the susceptibility of diabetic hearts to I-R injury have been inconsistent. While diabetic hearts were found to be more sensitive than the non-diabetic counterpart to anoxic or ischemic injury (11, 12, 27), it has also been reported that diabetic hearts showed a greater resistance to I-R injury, regardless of the abnormalities in cardiac function (13, 14, 26, 28). Whether or not the diabetic

Table 2. Effects of insulin treatment on ischemia-reperfusion (I-R) injury and its associated changes in tissue non-enzymatic antioxidants levels in diabetic rat hearts

	LDH (AU)	Contractile force recovery (%)	GSH (nmol/mg tissue)	V_C (μg/g tissue)	V_E (μg/g tissue)
Control	570 ± 28	89 ± 1	1.075 ± 0.015	40.6 ± 1.6	32.0 ± 1.5
Insulin	1283 ± 68[a]	85 ± 1	1.058 ± 0.008	47.4 ± 0.5[a]	21.6 ± 1.2[a]

Diabetic rats were treated with insulin twice daily as described in Materials and Methods. Isolated-perfused hearts prepared from control or insulin-treated diabetic rats were subjected to 20 min of ischemia followed by 20 min of reperfusion. The extent of LDH leakage and contractile force recovery, as well as myocardial levels of GSH, V_C and V_E were measured as described in Materials and Methods. The value given is the mean ± S.E.M., with n = 5. [a]Significantly different from the control diabetic group, $P<0.05$.

heart is more sensitive to I-R injury remains to be determined, but the finding of differential susceptibility between control and diabetic hearts to I-R challenge, as reported by the present study, may be related to the diabetes associated metabolic changes in the myocardium. In this regard, the increase in reliance on fatty acids as energy substrate is believed to be an important contributing factor to the development of biochemical changes in diabetic hearts (29, 30). This postulation is strengthened by the observation that the removal of inhibition of glucose oxidation by fatty acids through pharmacological interventions could ameliorate the contractile failure caused by mild myocardial ischemia in acute diabetic rats (31, 32). Under the present experimental conditions, the inability of isolated-perfused diabetic hearts to utilize glucose, which is the major fuel molecule present in the perfusate, for energy metabolism during post-ischemic reperfusion may at least in part contribute to the marked decrease in contractile force even after the prolonged period of reperfusion. This is also related to the observation that the reduction in the extent of I-R-induced LDH leakage was not associated with improvement in contractility in ischemic-reperfused diabetic hearts (Fig. 1: a and b).

In contrast to the slight and insignificant changes in tissue GSH level upon IR challenge in non-diabetic hearts, myocardial GSH level was increased during the early phase of reperfusion after 20 min of ischemia in diabetic hearts. While myocardial V_C was depleted to a large extent during the early phase of reperfusion after 20 min of ischemia, prolonged reperfusion of diabetic hearts, in contrast to the non-diabetic control, could increase myocardial V_C level. In addition, myocardial V_E level was greatly increased during the early phase of post-ischemic reperfusion in diabetic hearts. The results indicated differential changes in myocardial non-enzymatic antioxidant levels upon I-R challenge between control and diabetic hearts. The protection against I-R injury, as assessed by LDH leakage, in diabetic hearts was associated with increases in tissue GSH/V_C and V_E levels, with the effect on V_E being most prominent. Given that these non-enzymatic antioxidants act synergistically in cellular antioxidant defense (33, 34), the antioxidant response, as manifested by increases in tissue GSH, V_C and V_E levels under the oxidative stress condition, may contribute to the protection against I-R-induced injury in diabetic hearts observable during the early phase of reperfusion. In this connection, the decreased susceptibility of liver mitochondria from STZ-induced diabetic rats to oxidative damage has been found to be associated with an increase in mitochondrial V_E content (35). The observation of negative correlation of myocardial V_E and/or V_C , but not GSH, with the extent of I-R injury may be related to the ability of GSH to recycle V_E and V_C (36, 37). Myocardial GSH level therefore remained relatively insensitive to I-R-induced changes (Fig. 2a). The reversal effect of insulin treatment on diabetes-associated changes in hearts subjected to I-R challenge indicated that the aforementioned change in susceptibility of diabetic hearts to I-R injury was mainly caused by the diabetic state. The enhanced susceptibility of insulin-treated diabetic hearts to I-R injury was associated with a marked decrease in myocardial V_E level. The negative correlation between sensitivity to I-R injury and V_E level in diabetic hearts supports the ultimate role of myocardial V_E level in the antioxidant defense against I-R-induced oxidative stress.

It has been reported that metabolic changes occurring during the early phase of diabetes may “chemically” precondition the myocardium, rendering it more resistant to I-R injury (38). This is consistent with the observation of antioxidant response in diabetic hearts upon relatively mild I-R challenge, as reported in the present study. While the mechanism involved in the “chemical” preconditioning of diabetic heart remains unclear, it is possible that the exposure to oxidative stress arising from the diabetic state (16) may play a role in priming the myocardium in response to oxidative challenge such as I-R. However, prolonged exposure to metabolic changes, as in the case of chronic diabetes, could lead to the development of pathological conditions (39). By the same token, exposure of diabetic (preconditioned) hearts to severe oxidative stress condition, as in the case of 40 min of ischemia followed by 40-min reperfusion, could overwhelm the preconditioning effect, leading to a drastic increase in LDH leakage and impairment in contractile force.

In conclusion, isolated-perfused hearts prepared from short-term (2-week) diabetic rats appeared to be more resistant to I-R challenge during the early phase of post-ischemic reperfusion. The protracted reperfusion time course revealed a larger degree of I-R injury in diabetic hearts. The apparent protection against I-R injury during the early phase of post-ischemic reperfusion in diabetic hearts was associated with increases in myocardial GSH, V_C and V_E levels, with the effect on V_E being most prominent. Insulin treatment could reverse the diabetes-associated changes in susceptibility to myocardial I-R injury and antioxidant response. The results indicate that the myocardium isolated from short-term diabetic rat can produce a beneficial antioxidant response to I-R challenge, which may, in turn, be attributable to the decreased susceptibility to I-R injury observable during the early phase of reperfusion.

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