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Antidiabetic drug miglitol inhibits myocardial apoptosis involving decreased hydroxyl radical production and Bax expression in an ischaemia/reperfusion rabbit heart

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1 We examined whether antidiabetic drug miglitol could reduce ischaemia/reperfusion-induced myocardial apoptosis by attenuating production.

2 Japanese white rabbits were subjected to 30-min coronary occlusion followed by 4-h reperfusion with miglitol (10 mg kg^{-1}, i.v., $n=20$) or saline ($n=20$). The infarct area was determined by myoglobin staining, and the infarct size (IS) was expressed as a percentage of the area at risk. DNA fragmentation was assessed by TUNEL method and DNA ladder formation. The expression of Bcl-XL and Bax was detected by immunohistochemical analysis and Western blot analysis. Myocardial interstitial 2,5-DHBA levels, an indicator of hydroxyl radicals, were measured during 30-min ischaemia and 30-min reperfusion in the absence ($n=10$) or presence of miglitol (10 mg kg^{-1}, i.v., $n=10$) using a microdialysis technique.

3 The IS was significantly reduced in the miglitol group (22.4±3.4%, $n=10$) compared to the control group (52.8±3.5%, $n=10$). Miglitol significantly decreased the 2,5-DHBA level during ischaemia and reperfusion and suppressed the incidence of TUNEL-positive myocytes in the ischaemic region (from 10.7±3.4 to 4.1±3.0%) and the intensity of DNA ladder formation. Miglitol significantly decreased the incidence of Bax-positive myocytes in the ischaemic region (7.4±1.7 vs 13.7±1.9% of the control) and significantly attenuated the upregulation of Bax protein in the ischaemic regions (from 179±17 to 90±12% of sham). There was no difference in the expression of Bcl-XL between the two groups.

4 These data suggest that miglitol reduces myocardial apoptosis by attenuating production of hydroxyl radicals and suppressing the upregulation of the expression of Bax protein.
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Keywords: Myocardial infarction; apoptosis; miglitol; ischaemia; α-glucosidase inhibitor; hydroxyl radicals

Abbreviations: AAR, area at risk; DBP, diastolic blood pressure; 2,5-DHBA, 2,5-dihydroxybenzoic acid; IS, infarct size; LV, left ventricle; miglitol, *N*-hydroxyethyl-1-deoxynojirimycin; SBP, systolic blood pressure; TUNEL, *in situ* nick end labelling

Introduction

Brief episodes of ischaemia and reperfusion before a subsequent prolonged ischaemia precondition the myocardium and reduce myocardial infarct size (IS). This phenomenon is known as ischaemic preconditioning (Murry *et al*., 1986). One of the mechanisms of ischaemic preconditioning is the reduction of energy demand, as evidenced by improved preservation of ATP and the attenuation of lactate and H^+ accumulation during the subsequent sustained ischaemic insult (Kida *et al*., 1991), which have been considered to be due to pre-ischaemic glycogen depletion by ischaemic preconditioning (Jennings *et al*., 1991). *N*-hydroxyethyl-1-deoxynojirimycin (miglitol), an α-1,6- and α-1,4-glucosidase inhibitor, is a new human antidiabetic agent and currently used clinically for the treatment of diabetes mellitus. It exerts an antihyperglycemic effect by blocking α-1,4-glucosidase in the intestine (Bollen & Stalmans, 1989). Recently, we found that miglitol markedly reduced the myocardial IS in a rabbit model of 30 min ischaemia and 48 h reperfusion (Minatoguchi *et al*., 1999). We reported that the IS-reducing effect of miglitol was related to the inhibition of glycogenolysis during ischaemia (Minatoguchi *et al*., 1999). On the other hand, it has been reported that oxygen-free radicals such as superoxide anion and hydroxyl radicals play an important role in ischaemia and reperfusion injury (Bolli, 1988), and the blockade of oxygen-free radicals during reperfusion reduces myocardial IS (Horwitz *et al*., 1994; Hashimoto *et al*., 2001). Furthermore, ischaemia and reperfusion is reported to increase the incidence of myocardial apoptosis, which is regulated by Bcl-XL and Bax, an inhibitor and a promoter of apoptosis, respectively (Chao & Korsmeyer, 1998; Cook *et al*., 1999). At present, the effects of miglitol on these factors are still unknown. The purpose of the present study was to investigate the effect of miglitol on myocardial

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apoptosis induced by ischaemia and reperfusion and to examine the contribution of oxygen-free radicals and Bcl-XL and Bax to the effect of miglitol in a rabbit heart without collateral circulation.

Methods

In this study, all rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institute of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Animal selection

Male Japanese white rabbits (Chubu-Kagaku-Shizai Co., Nagoya, Japan) each weighing 2–2.5 kg were used. None of the rabbits had any clinically evident infections.

Surgical preparation

Rabbits were anaesthetized with sodium pentobarbital (30–40 mg kg^{-1}, i.v.) and additional doses were administered when required throughout the experiment. They were intubated and ventilated with room air supplemented with a low flow of oxygen by a mechanical ventilator (tidal volume, 20–30 ml; respiratory rate, 20–30 min^{-1}; model SN-480-5; Shinano, Tokyo, Japan). Serial blood gas analysis was performed, and ventilatory parameters were adjusted to keep the arterial blood gas within the physiological range. Surgery was performed under sterile conditions. The right carotid artery and jugular vein were cannulated to monitor peripheral arterial pressure and to administer drugs or saline. Rabbits were then administered heparin (500 U kg^{-1}). A thoracotomy was performed in the third intercostal space, and the heart was exposed after excising the pericardium. A 4–0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle of the anterolateral surface of the left ventricle (LV). A small vinyl tube was passed into both ends of the suture, and the coronary branch was occluded by pulling the snare, which was then fixed by clamping the tube with a mosquito haemostat. Myocardial ischaemia was confirmed by regional cyanosis and electrocardiographic change. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snare. After the initial preparation but before coronary occlusion, the animals were assigned randomly to groups. All rabbits were allowed to rest for 20 min after completion of the surgical preparation before the start of the protocol. The rabbits underwent a 30-min occlusion of an anterolateral branch of the coronary artery, followed by a 4-h reperfusion (protocol 1) or a 30-min reperfusion (protocol 2).

Experimental protocols (Figure 1)

Experimental protocol 1 was used to investigate the effect of miglitol on the IS, *in situ* nick end labelling (TUNEL)-positive myocytes, DNA fragmentation by agarose gels, expression of Bcl-xL and Bax by immunohistochemical analysis and by Western blot analysis. Experimental protocol 2 was used to investigate the effect of miglitol on myocardial interstitial 2,5-DHBA levels, an indicator of hydroxyl radicals.

IS (protocol 1)

The animals were subjected to 30 min of ischaemia and 4 h of reperfusion. The control group ($n=10$) was injected with 1 ml saline 30 min before ischaemia. The miglitol group ($n=10$) was administered a 10 mg kg^{-1} i.v. bolus of miglitol 30 min before ischaemia. The animals were killed at 4 h after reperfusion. At the end of the study, the rabbits were heparinized (500 U kg^{-1})

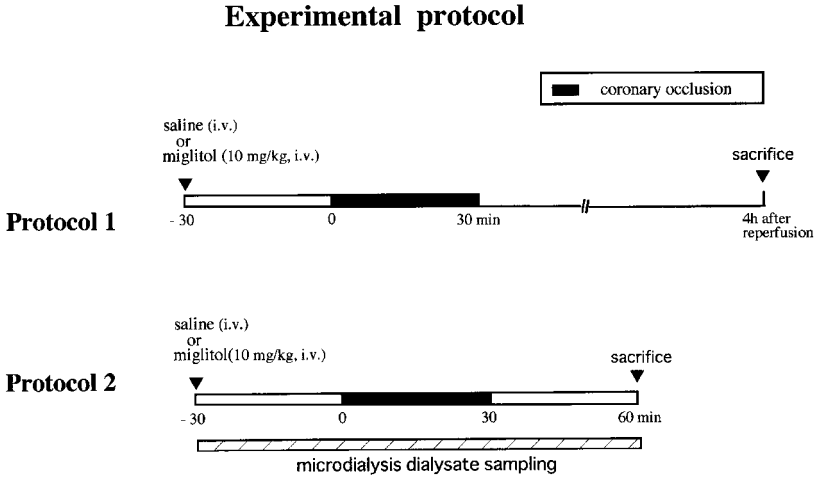

Figure 1 Experimental protocols. Protocol 1: control group ($n=20$) – 30 min coronary occlusion followed by 4 h of reperfusion with saline injection 30 min before ischaemia; miglitol group ($n=20$) – 30 min coronary occlusion followed by 4 h of reperfusion with 10 mg kg^{-1} of miglitol injection 30 min before ischaemia. Half of each group was included for IS and immunohistochemical analysis, and the other half of each group for Western blot analysis. Protocol 2: control group ($n=10$) – 30 min coronary occlusion followed by 30 min of reperfusion with saline injection 30 min before ischaemia; miglitol group ($n=10$) – 30 min coronary occlusion followed by 30 min of reperfusion with 10 mg kg^{-1} of miglitol injection 30 min before ischaemia.

and killed by an overdose of pentobarbital. Each heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded and 1% Evans blue dye was injected from the aorta at 80 mmHg to determine the area at risk (AAA). The LV was sectioned into seven slices parallel to the atrio-ventricular ring. After fixation in 10% formalin, both atria and the right ventricular free walls were removed. The LV was cut into six or seven transverse slices, each of which was then embedded in paraffin and sectioned by a microtome (4 μm thickness). Serial microscopic sections were cut from the apical surface of each block and stained with haematoxylin-eosin and myoglobulin for the determination of infarct area. Using an indirect immunoperoxidase method, immunohistochemical staining was performed using monoclonal anti-human myoglobin (Sigma) at 1 : 100. The myoglobin antibody immunohistochemical method using the paraffin-embedded tissue fixed with formalin is more valuable for the detection of early myocardial infarction (Fujiwara *et al*., 1988). The risk areas and infarcted areas were traced on each LV slice and the mean values for each heart were obtained from four serial apex-side to base-side slices in which infarct occurred, and the IS was obtained as a percentage of the risk area for each heart. The determination of IS was performed by an investigator blinded to treatment.

Tissue preparation (protocol 1)

The other rabbits were subjected to 30 min of ischaemia and 4 h of reperfusion. The control group ($n = 10$) was injected with 1 ml saline 30 min before ischaemia. The miglitol group ($n = 10$) was administered a 10 mg kg^{-1} i.v. bolus of miglitol 30 min before ischaemia. The animals were killed at 4 h after reperfusion. The LV was separated into risk and nonrisk areas and each specimen was immediately frozen in liquid nitrogen and stored at -83°C for further analysis.

Detection of TUNEL-positive myocytes (protocol 1)

Fixed transverse ventricular slices were embedded in paraffin, and 4 μm thick sections were deparaffinized by washing in 100% xylene and a descending ethanol series (from 100% ethanol two times to 80% ethanol once and 60% ethanol once). The sections were stained with haematoxylin. DNA fragments were determined using an ApopTag *in situ* apoptosis detection kit (ApopTag, Oncor, U.S.A.). The DNA nick was labelled according to the manufacturer's instructions, which were based on the method described by Schmitz *et al*. (1991). After TUNEL, the sections were counterstained with haematoxylin.

Cardiomyocytes in the infarcted area were counted under light microscopic analysis. In each specimen, cardiomyocytes with counterstained nuclei were counted in 60 random high-power fields (400) from the endocardial portion in the infarcted areas. Myocytes in which the nucleus was obviously labeled with diaminobenzidine were defined as TUNEL-positive. The percentage of TUNEL-positive myocytes from approximately 3000 myocytes with a nucleus in the infarcted area was then calculated. This evaluation was performed independently by two persons who were unaware of the experimental protocol.

DNA fragmentation by agarose gel electrophoresis (protocol 1)

In a separate series of experiments, transmural myocardial samples ($n = 5$, each) from risk and nonrisk areas were frozen in liquid nitrogen and stored at -80°C until further analysis. Myocardial DNA was extracted from 70–80 mg of myocardium by a nucleic acid extraction kit (IsoQuick®, Microprobe, U.S.A.). The extracted DNA (10 mg) was loaded on 2% agarose gel containing ethidium bromide and electrophoresed on a 100 V in TBE buffer (0.04 mol l^{-1} Tris, 0.04 mol l^{-1} borate acid, 2 mmol l^{-1} EDTA, pH 8.0). The gels were photographed under UV light.

Detection of the expression of Bcl-XL and Bax by immunohistochemistry (protocol 1)

A mouse monoclonal anti-Bcl-XL IgG1 antibody (sc-8392, Santa Cruz Biotechnology, CA, U.S.A.) as the primary antibody was used for Bcl-XL immunohistochemical staining. Indirect immunoperoxidase staining was performed as described, with some modifications. Deparaffinized tissue sections were placed in a glass jar filled with 10 mmol l^{-1} citrate buffer, pH 6.0, and placed in a 500-W microwave oven twice for 5 min. Intrinsic peroxidase activity was inhibited by 0.3% hydrogen peroxide in methanol for 30 min, and nonspecific binding was blocked with normal goat serum. The primary antibody was diluted 1 : 20 and incubated with the sections overnight at 4°C. The second antibody, peroxidase-conjugated F(ab′)2 fragment goat anti-mouse IgG (H + L) (Jackson Immunoresearch Laboratories, Inc., PA, U.S.A.), was incubated with the sections at a dilution of 1 : 500 for 40 min at room temperature. Sections were then stained with 0.4 mg ml^{-1} 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) and 0.006% hydrogen peroxide in 50 mmol l^{-1} Tris–HCl, pH 7.4, buffer for 5 min at room temperature. Between each step, the sections were washed with 10 mmol l^{-1} sodium PBS (Na_2HPO-$12H_2O$ 57.4 g, NaH_2PO_4-$2H_2O$ 6.6 g, NaCl 34.0 g in 4 l of distilled water). Finally, the sections were counterstained with haematoxylin.

A mouse monoclonal anti-Bax IgG2b antibody (sc-7480, Santa Cruz Biotechnology, CA, U.S.A.) as the primary antibody was used for Bax immunohistochemical staining. The deparaffinized thin sections were treated by microwave irradiation twice at 500 W for 5 min with 10 mmol l^{-1} citrate buffer (pH 6.0). Intrinsic peroxidase activity was inhibited by 0.3% hydrogen peroxide in methanol for 30 min, and nonspecific binding was blocked with normal goat serum. Thereafter, the sections were soaked for 30 min in 5 μg ml^{-1} saponin in distilled water at room temperature. The primary antibody was diluted 1 : 500 and incubated with the sections overnight at 4°C. The sections were then incubated with peroxidase-conjugated F(ab′)2 fragment goat anti-mouse IgG (H + L) for 40 min. For the negative control, primary antibody was omitted. After being soaked in 0.5% Triton X-100/PBS, sections were stained with DAB solution and counterstained with haematoxylin.

Determination of the expression of Bcl-XL and Bax protein by Western blot (protocol 1)

Frozen samples were weighed and homogenized in 5 v of buffer containing sucrose 300 mM, HEPES 4 mM, EGTA

2 mM, phenylmethylsulphonyl fluoride(PMSF) 1 mM and leupeptin 20 μM using a polytron homogenizer at the maximum speed in five 5-s bursts. The homogenates were centrifuged at 14,000 $\times g$ for 30 min at 4°C. The protein concentrations in the resulting supernatant samples were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, U.S.A.). After heating at 95°C for 5 min, the proteins (30 μg) present in each sample fraction were separated by SDS–PAGE on 8.0% gels and then electrophoretically transferred to 0.45 μm PDGE membranes using a Semi-Dry Transfer Cell (Bio-Rad Laboratories). The transfer buffer contained (mM) Tris–HCl 25 and glycine 192 in 20% methanol. The blots were initially blocked overnight with 5% milk in buffer containing Tris–HCl 20 mM (pH 7.4), NaCl 137 mM, 0.05% Tween-20, and then incubated for 1 h with anti-Bcl-XL antibody diluted 1 : 250 or anti-Bax antibody diluted 1 : 250, both from Santa Cruz Biotechnology, CA, U.S.A. After washing, the blots were incubated for 1 h at room temperature with a peroxidase-linked, goat anti-mouse secondary antibody (1 : 1000 dilution). The internal control was monoclonal anti-actin antibody (Sigma, St Louis, U.S.A.) diluted 1 : 250. The bound antibody was then visualized using an enhanced chemiluminescence (ELC) kit (Amersham). Bcl-XL and Bax were quantified densitometrically using suitable autoradiographs.

Measurement of myocardial interstitial hydroxyl radicals (protocol 2)

In all, 20 rabbits were assigned to investigate the effect of miglitol on the level of myocardial interstitial 2,5-DHBA, an indicator of hydroxyl radical, during 30-min ischaemia and 30-min reperfusion. A microdialysis probe (PNF 1700; Asahi Medical, Tokyo, Japan; 20 mm length, 0.31 mm OD, 0.2 mm ID; transverse type, 50,000 MW cutoff) for dialysate sampling was implanted in the risk area of the myocardium, which was served by the anterolateral coronary artery along the axis of the ventricular fibres and reached from the epicardial outer layer to the endocardial inner layer of the myocardium. Probe placement was confirmed at autopsy. The microdialysis probe was perfused with 1 mM salicylic acid dissolved in Ringer's solution at a rate of 10 μl min^{-1}. After a 60-min rest following the completion of instrumentation, the dialysate was sampled during 30 min pre-ischaemia, during 30 min ischaemia and during 30 min reperfusion at an interval of 10 min in the presence of saline ($n = 10$) or miglitol (10 μg kg^{-1}, i.v., at 30 min before ischaemia, $n = 10$). Dialysate samples were frozen at −83°C until further analysis. The measurement of hydroxyl radical was based on the reaction between salicylic acid and hydroxyl radical; 1 mM salicylic acid can trap approximately 10% of theoretically generated hydroxyl radical, producing 2,3-dihydroxybenzoic acid (DHBA), 2,5-DHBA and catechol as the derivatives in proportions of 49, 40 and 11%, respectively (Grootvelt & Halliwell, 1986). In the present study, we used 2,5-DHBA as an indicator of hydroxyl radical production because of its high specificity for hydroxyl radical (Takemura *et al*., 1992). The column used in the present study was an MCM C18 column (6 × 250; 5-120A; MC Medical Inc., Tokyo, Japan). The 2,5-DHBA was measured using high-performance liquid chromatography coupled with electrochemical detection, as described previously (Chen *et al*., 2003).

Statistical analysis

Values are expressed as the group means ± s.e.m. To compare the group means, one-way analysis of variance (ANOVA) was performed, and if the ANOVA was significant, a Scheffe's *F*-test was performed to assess which group was significantly different. Differences with a value of $P < 0.05$ were considered significant.

Results

Hamodynamic parameters

Table 1 shows the hamodynamic parameters. There was no significant difference in systolic blood pressure, diastolic blood pressure or heart rate among the two groups in protocols 1 and 2.

Infarct size

Figure 2 shows the AAA as a percentage of LV and the IS as a percentage of the AAA. There was no significant difference in the mean percentages of AAA as a percentage of LV between the control group (43.2 ± 2.6%) and the miglitol group (43.7 ± 4.6%). The IS as a percentage of AAA was significantly reduced in the miglitol group (22.4 ± 3.4%) compared with the control group (52.8 ± 3.5%).

TUNEL-positive myocytes

Figure 3 shows TUNEL-positive myocytes in the control and miglitol groups. The TUNEL-positive nuclei were observed in the infarcted area but not in the nonrisk area. The percentage of myocytes with DNA fragmentation, as assessed by TUNEL in the infarcted area, was significantly reduced in the miglitol group (4.1 ± 3.0%) compared with the control group (10.7 ± 3.4%).

DNA fragmentation by agarose gel electrophoresis

A series of DNA fragments showing size ranges in multiples of 18–200 bp units is called a ladder pattern, which indicates apoptotic internucleosomal DNA fragmentation. DNA ladder formation pattern, which is characteristic of DNA fragmentation, was distinctly visible in agarose gels of DNA from the myocardium of the ischaemic area in the control group (Figure 4). The treatment with miglitol diminished the intensity of the ladder formation (Figure 4).

Immunohistochemistry of Bcl-XL and Bax proteins

There was no significant difference in the incidence of Bcl-XL positive myocytes in the infarcted area between the miglitol group (38.8 ± 2.6%) and control group (31.5 ± 3.7%) (Figure 5). In contrast, the incidence of Bax-positive myocytes was significantly decreased in the miglitol group (7.4 ± 1.7%) compared with the control group (13.7 ± 1.9%) (Figure 6).

Table 1 Hemodynamic parameters

	Baseline	*Before occlusion*	*20 min occlusion*	*20 min reperfusion*
Protocol 1				
SBP (mmHg)				
Control ($n=20$)	107±3	105±4	95±4	85±4
Miglitol ($n=20$)	105±4	104±3	94±4	84±3
DBP (mmHg)				
Control ($n=20$)	87±4	86±4	75±5	70±4
Miglitol ($n=20$)	86±5	85±4	76±4	71±3
Heart rate (beats min^{-1})				
Control ($n=20$)	258±6	257±7	244±6	235±6
Miglitol ($n=20$)	256±7	248±8	237±7	229±8
Protocol 2				
SBP (mmHg)				
Control ($n=20$)	108±6	106±7	97±6	86±7
Miglitol ($n=20$)	107±7	106±6	95±7	85±6
DBP (mmHg)				
Control ($n=20$)	88±6	87±7	76±6	72±5
Miglitol ($n=20$)	89±7	86±6	78±5	73±4
Heart rate (beats min^{-1})				
Control ($n=20$)	259±7	258±8	245±7	236±7
Miglitol ($n=20$)	258±8	249±7	239±8	232±6

SBP = systolic blood pressure, DBP = diastolic blood pressure.

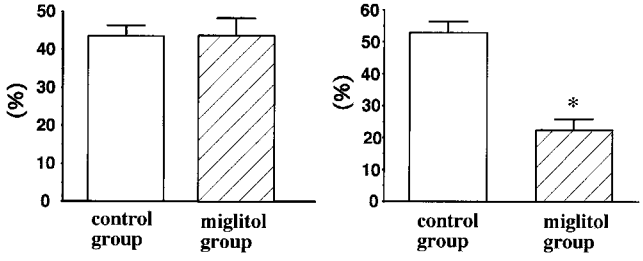

Figure 2 AAA as a percentage of LV and the IS as a percentage of AAA in the control group ($n=10$) and miglitol group ($n=10$) *$P<0.05$ vs control group.

Western blot analysis of Bcl-XL and Bax proteins

As shown in Figure 7, there was no significant difference in the expression of Bcl-XL protein in the ischaemic area of LV between the miglitol group (110±16% of sham) and control group (95±8% of sham). However, the expression of Bax protein was significantly upregulated in the infarcted area of the control group (178±17%) as compared to the sham group. However, this upregulation of the expression of Bax protein was significantly inhibited in the miglitol group (90±12% of sham).

Effect on myocardial interstitial 2,5-DHBA level

As shown in Figure 8, myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radical, were significantly increased at 20 and 30 min after the start of coronary occlusion and at 10 min after the start of reperfusion compared with the pre-ischaemic period. Pretreatment with miglitol significantly attenuated the increase in myocardial interstitial 2,5-DHBA levels during ischaemia and reperfusion.

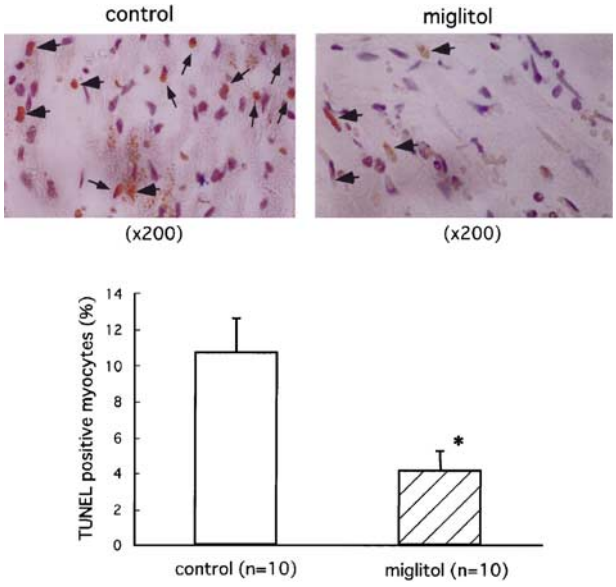

Figure 3 Upper: Photomicrograph (light microscopic TUNEL analysis) of myocardium from the control and miglitol groups subjected to 30 min ischaemia followed by 4 h reperfusion (× 400). Brown TUNEL-positive nuclei are seen in infarcted myocytes (arrows). Lower: The incidence of TUNEL-positive myocytes in the control ($n=10$) and miglitol ($n=10$) groups. *$P<0.05$ vs control group.

Discussion

The present data demonstrated that (1) miglitol significantly reduced the myocardial IS, the incidence of TUNEL-positive myocytes and the intensity of DNA ladder formation, (2) miglitol decreased the interstitial levels of 2,5-DHBA, an indicator of hydroxyl radicals, during ischaemia and reperfusion, and suppressed the upregulation of the expression of Bax

Figure 4 Agarose gel electrophoresis of DNA extracted from the ischaemic area of the myocardium. Lane 1: sham; lane 2: control group; lane 3: miglitol group. DNA ladder formation was observed in the control group, but not in the miglitol group.

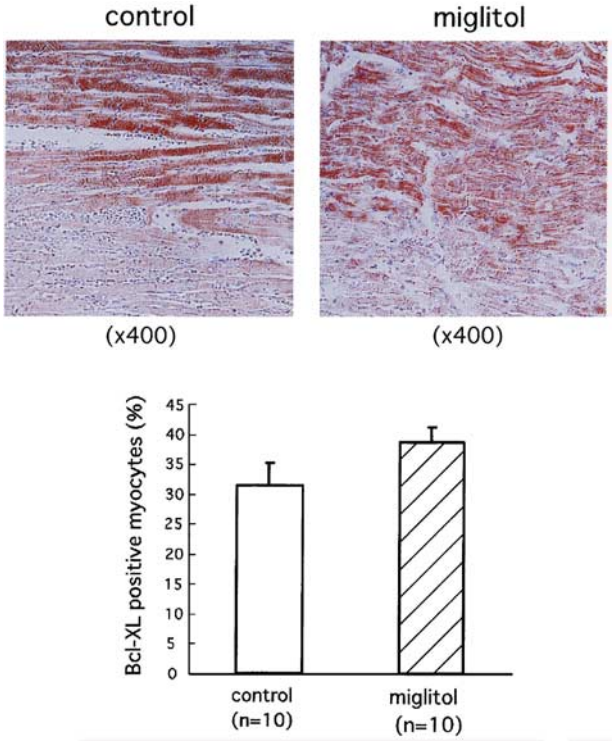

Figure 5 Immunohistochemistry of Bcl-xL proteins.

protein in the ischaemic area of LV. This is the first study to show that miglitol reduces myocardial apoptosis by inhibiting production of hydroxyl radicals and suppressing the expression of Bax.

Production of reactive oxygen species during the reperfusion period has been regarded as an important factor in reperfusion injury (Bolli, 1988). Reactive oxygen species such as superoxide and hydroxyl radicals are suggested to be predominant mediators of reperfusion-induced necrotic cell death. Among the reactive oxygen species, the hydroxyl radical is highly reactive and plays a critical role in post-ischaemic myocardial

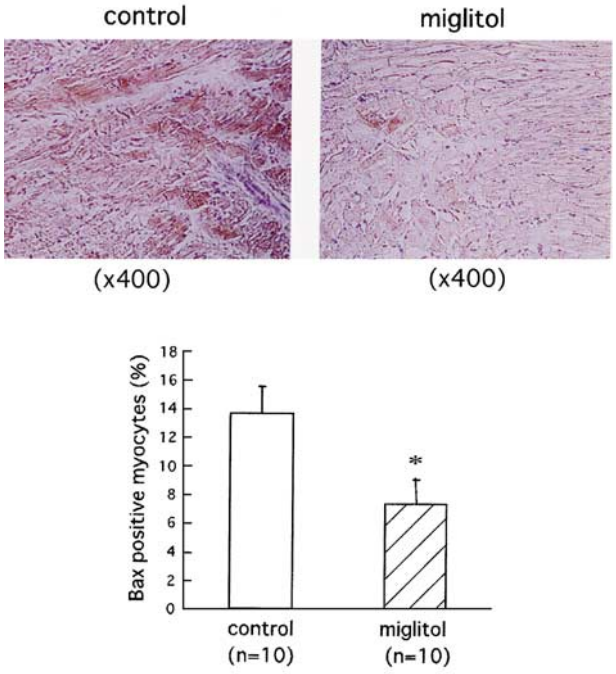

Figure 6 Immunohistochemistry of Bax proteins. $*P<0.05$ vs control group.

damage during reperfusion (Portz *et al*., 1989). In the present study, we investigated whether miglitol reduces the myocardial interstitial levels of hydroxyl radical, using a microdialysis technique. We found that hydroxyl radicals were generated in the interstitium during both ischaemia and reperfusion, and that miglitol reduced the level of hydroxyl radicals in the myocardium during ischaemia and reperfusion. This suggests that one of the mechanisms of miglitol for reducing the IS may be related to the reduction of hydroxyl radical production during ischaemia and reperfusion. It may be considered that the formation of the hydroxyl radicals cannot occur during ischaemia in the absence of oxygen. However, it has already been reported that myocardial free radicals are generated even during ischaemia at 13 min coronary occlusion, as assessed by electron magnetic resonance (Bolli, 1991). It has also been reported that during ischaemia the components of the mitochondrial electron transport chain become reduced (Freeman & Crapo, 1982; McCord, 1988), allowing an increase of electron leakage from the respiratory chain which, in turn, will react with residual molecular oxygen, leading to the formation of oxygen-free radicals (Ferrari *et al*., 1993).

We have previously reported that miglitol can inhibit the α-1,6-glucosidase glycogen debranching enzyme, and actually inhibited the glycogenolysis during 30 min of ischaemia in an *in vivo* rabbit heart (Minatoguchi *et al*., 1999). However, since it has been reported that hearts are also able to utilize energy substrates such as fatty acids (Stanley *et al*., 1997) other than glucose, a change in supply of one particular energy substrate may not have a profound effect on the overall rate of oxidative respiration and consequent free radical production. Therefore, further investigation on the mechanisms of reduced production of hydroxyl radicals by miglitol would be warranted.

It has been reported that reactive oxygen species released from myocytes after ischaemia and reperfusion may trigger both necrosis and apoptosis (Hare, 2001). Free radical

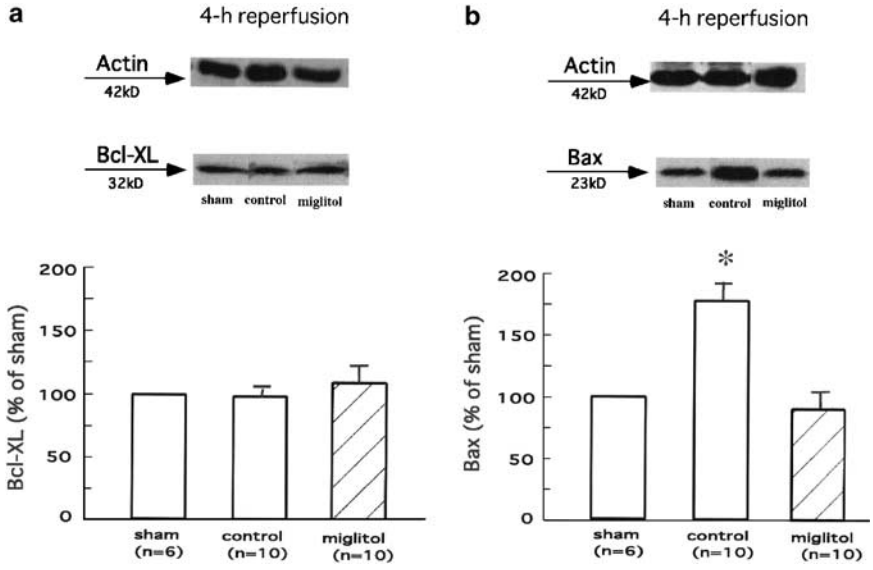

Figure 7 Western blot for expression of Bcl-xL and Bax proteins in the ischaemic area of the myocardium in the sham ($n=6$), control ($n=10$) and miglitol ($n=10$) groups. *$P<0.05$ vs sham and control.

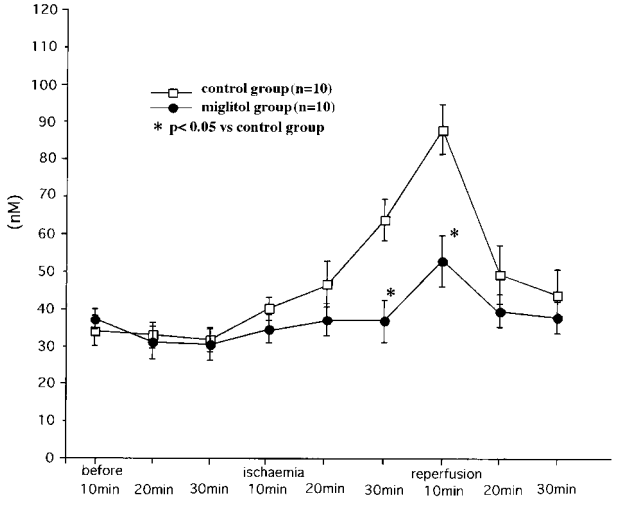

Figure 8 Myocardial interstitial 2,5-DHBA levels, an indicator of hydroxyl radicals during 30 min ischaemia and 30 min reperfusion in the control ($n=10$) and miglitol ($n=10$) groups. *$P<0.05$ vs control group.

scavengers have been reported to inhibit the appearance of apoptosis, which suggests reactive oxygen species as triggers of apoptosis (Yang *et al*., 1996; Ambrosio *et al*., 1998). In the present study, the incidence of TUNEL-positive myocytes and the intensity of DNA ladder formation were significantly decreased in the miglitol group compared with the control group, suggesting that miglitol inhibited apoptosis of myocytes. Therefore, it is likely that miglitol inhibited apoptosis of myocytes by reducing the burst of oxygen-free radicals such as hydroxyl radicals.

Apoptosis is governed by a member of the regulating genes mediated by apoptotic signals. Members of the Bcl-2 family that act as inhibitors of apoptosis include Bcl-2, Bcl-XL and Bcl-w, and those that act as promoters of apoptosis include Bax, Bad, Bak and Bcl-Xs. The ratio of the antiapoptotic proteins and proapoptotic proteins is critical in determining whether the cell survives or dies. Cytochrome *c* released from mitochondria has been reported to bind to Apaf-1 to activate caspase 9, which can cleave and activate caspase 3, leading to the formation of various death substrates that produce apoptosis (Sabbah, 2000). Bcl-2 can interfere with cytochrome *c* release and can also suppress the actions of Apaf-1 and protect from apoptosis (Golstein, 1997). It has been reported that ischaemia without reperfusion did not induce apoptosis or alter the appearance of apoptotic regulating proteins (Zhao *et al*., 2000). However, ischaemia followed by reperfusion induced a time-dependent reduction in the expression of Bcl-2 protein and increase in the expression of Bax and p53 proteins (Zhao *et al*., 2001). Recently, Nakamura *et al*. (2000) reported that ischaemic preconditioning reduced ischaemia/reperfusion-induced myocardial apoptosis by downregulating the expression of Bax, but not by changes in expression of Bcl-2 in a rat model of 30-min ischaemia and 180-min reperfusion. Yamamura *et al*. (2001) reported that myocardial apoptosis induced by insulin-like growth factor-I was associated with increased Bcl-XL/Bax ratio. In the present study, miglitol significantly suppressed the overexpression of Bax in the ischaemic area, whereas 30 min ischaemia and 180 min reperfusion by itself did not alter Bcl-XL expression and treatment with miglitol did not affect the expression of Bcl-XL. Therefore, the antiapoptotic effect of miglitol may be attributed to the suppression of upregulation of Bax expression and an increase in the Bcl-XL/Bax ratio.

It is well known that diabetes mellitus is one of the most important risk factors of coronary artery disease and there are many patients with combined diabetes mellitus and coronary artery disease. If the patients with diabetes mellitus were being treated with miglitol, an antidiabetic drug, these patients would be resistant against ischaemic event. Further clinical investigations are warranted.

In conclusion, treatment with miglitol reduces myocardial apoptosis by decreasing the production of hydroxyl radicals during ischaemia and reperfusion and suppressing the upregulation of Bax expression after reperfusion.

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