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ATTENUATION OF MYOCARDIAL REPERFUSION INJURY BY REDUCING INTRACELLULAR CALCIUM OVERLOADING WITH DIHYDROPYRIDINES

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Abstract—The effects of three different dihydropyridine (DHP) calcium channel antagonists, nisoldipine, nimodipine, and nifedipine, on myocardial ischemic and reperfusion injury were studied using isolated rat hearts subjected to ischemia and reperfusion. Hearts were perfused with Krebs–Henseleit bicarbonate buffer containing 0, 4, 16, 64 and 100 nM concentrations of the above dihydropyridines for 15 min. Global ischemia was then induced by terminating the aortic flow for 30 min at 37°, followed by 30 min of reperfusion. Left ventricular (LV) functional (LV developed pressure, its first derivative and coronary flow) and biochemical parameters (creatine kinase release) were monitored prior to ischemia and during reperfusion. In separate group of hearts, intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was monitored with an intracellular calcium analyzer using a fluorescent Ca^{2+} indicator (Fura-2 AM). Tissue Ca^{2+} was also measured by atomic absorption spectroscopy after perfusing the hearts with ion-free cold buffer to wash out extracellular Ca^{2+}. Significant recovery of the coronary flow was observed in all hearts treated with a high concentration (100 nM) of DHPs compared with the control group ($P < 0.05$), while a lower dose of nisoldipine (16 nM) and nifedipine (64 nM) also improved the coronary flow effectively. Reduction of myocardial creatine kinase release and improvement of the recovery of LV developed pressure, dp/dt_{max}, were achieved by DHPs in a concentration-dependent manner. A higher concentration of DHPs also decreased the formation of myocardial thiobarbituric acid reactive substances, although these compounds did not possess direct free radical scavenging effects *in vitro*. Tissue Ca^{2+} content was reduced significantly in treated groups. The rise of $[Ca^{2+}]_i$ during ischemia and reperfusion appeared to be attenuated by these DHPs. The concentration–response study of the three DHPs showed the effective concentrations for reducing $[Ca^{2+}]_i$ to be 16, 64 and 100 nM nisoldipine, nifedipine and nimodipine, respectively, in this experimental setting. The above results indicate that pretreatment with DHPs can attenuate the myocardial reperfusion injury by modulating Ca^{2+} overloading and decreasing the susceptibility of the membrane to free radical attack.

The therapeutic efficacy of revascularization after acute myocardial infarction depends on the recovery of the ischemic myocardium [1, 2]. Because such reperfusion can aggravate an existing ischemic injury, modification of reperfusion is of prime importance in facilitating myocardial salvage [3]. Although the mechanism of reperfusion injury remains unknown, several interrelating factors including oxygen free radicals [4], membrane phospholipid degradation [5], and intracellular Ca^{2+} overloading [6] have been implicated in the pathogenesis of myocardial reperfusion injury. Among these events, intracellular Ca^{2+} accumulation is perhaps the most critical factor in determining the biochemical basis of ultimate cell death [6, 7]. Excessive Ca^{2+} influx into the cell leads to a variety of changes at the cellular and molecular levels that ultimately cause irreversible cell injury [6]. Inhibition of Ca^{2+} entry thus represents an effective therapeutic intervention for myocardial salvage. A wide variety of Ca^{2+} channel antagonists have been found to attenuate the myocardial reperfusion injury. For example, verapamil [8], nifedipine [9], diltiazem [10], and lidoflazine [11] were all found to reduce myocardial reperfusion injury. Although the chemical structures of these Ca^{2+} blockers vary greatly, they all share a common mechanism, i.e. blockade of the slow inward Ca^{2+} channel [8–11]. However, the exact mode, timing, and efficacy of these Ca^{2+} blockers against reperfusion injury remain controversial.

Recently, a new dihydropyridine (DHP§), nisoldipine, has been shown to be more potent than nifedipine in antagonizing Ca^{2+} *in vitro* [12, 13]. This study sought to examine the effects of nisoldipine on myocardial ischemic and reperfusion injury, and to compare these effects with those of nifedipine and nimodipine [14] (a DHP with high affinity for the cerebrovascular system), simultaneously examining their efficacy in reducing Ca^{2+} overloading by two independent techniques to measure the intracellular Ca^{2+} in the isolated rat heart.

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§ Abbreviations: DHP, dihydropyridine; LV, left ventricle; LVEDP, left ventricle end-diastolic pressure; CF, coronary flow; KHB, Krebs–Henseleit bicarbonate buffer; CK, creatine kinase; and TBARS, thiobarbituric acid reactive substances.

MATERIALS AND METHODS

Perfused heart preparation. Male Sprague–Dawley rats weighing 250–300 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 85 mg/kg). The hearts were removed and quickly mounted on a non-circulating Langendorff perfusion column. Retrograde perfusion was established at a pressure of 100 cm H_2O with oxygenated normothermic Krebs–Henseleit bicarbonate buffer (KHB). KHB buffer consisted of the following ion concentrations (in mM): NaCl, 119.0; $NaHCO_3$, 25.0; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; and glucose, 11.0. A compliant latex balloon catheter filled with saline was inserted into the left ventricle (LV) through the left atrium to measure the LV isovolumic pressure, and the volume of the balloon was adjusted to produce a LV end-diastolic pressure of 8–10 mm Hg at baseline condition. The LV pressure catheter was connected to a pressure transducer (Statham P23 I.D.). The pressure signal was analyzed further by a differentiator to obtain its first derivative (dp/dt). The tracings were recorded in a Honeywell recorder. Coronary flow (CF) was measured by timed collection of coronary venous effluent.

Hearts were allowed to equilibrate for 10 min at 37° with non-recirculating KHB buffer. Then the hearts were perfused with recirculating KHB buffer (total volume was 50 mL) containing either nisoldipine, nimodipine, or nifedipine (0, 4, 16, 64, 100 nM each) for 15 min. The DHPs were dissolved in ethanol and further diluted with KHB to the desired concentration; the final concentration of ethanol in the recirculating KHB was 0.001%. Control hearts also received the same concentration of ethanol in the KHB buffer. The retrograde aortic flow was then terminated, and the heart was made ischemic for 30 min in a thermostatic chamber at 37°. Reperfusion was followed for 30 min using the same recirculating KHB containing DHPs. LV pressure and CF were measured prior to ischemia and at different stages of reperfusion. Perfusates were sampled and creatine kinase (CK) activity in the recirculating perfusate was determined; this activity represents the accumulation of CK over time. At the end of each experiment, the heart was excised at the atrioventricular junction and frozen in liquid nitrogen.

Monitoring of intracellular Ca^{2+} *transient* ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ was measured in a separate group of experiments as described below. Fura-2 acetoxymethyl ester (Fura-2 AM, Sigma Chemical Co., St. Louis, MO) was solubilized in a mixture of dimethyl sulfoxide/Cremophor EL (2.5%, w/v) (Sigma Chemical Co.), 0.1 mL of which was added to 50 mL of KHB solution to give a final concentration of 1 μM. Perfusion with Fura-2 AM containing KHB solution continued for 15 min; this was followed by a 15-min washout with normal KHB solution prior to the baseline measurement of Fura-2 fluorescence. The experimental system used a calcium analyzer (CAF-100, Jasco, Inc., Baltimore, MD) equipped with a dual wavelength spectrofluorimeter for exciting and detecting Fura-2 fluorescence. Fluorescence excitation was provided by a Xenon lamp. Illumination passed through a filter wheel which had two transmissive sectors, one that transmits primarily 340 nm light and another that transmits primarily 380 nm light (each with a band pass of 11 nm). The filter wheel was operated at 48 revolutions/sec by a rapidly spinning motor. The excitation light was then diverted onto a circular region of the left ventricular epicardium in a diameter of 1 cm via a dichroic mirror. Fluorescence then passed through an emission filter centered at 500 nm (a band pass of 12 nm) before reaching a photomultiplier. Photomultiplier output was entered into an electronic ratio circuit and the fluorescence ratio, F_{340}/F_{380}, was displayed on a strip chart recorder. An electrocardiogram was monitored simultaneously by using epicardial electrodes and recorded. Different concentrations of dihydropyridines were administered prior to the induction of global ischemia, and the $[Ca^{2+}]_i$ was monitored continuously in the control and treated groups.

Measurement of tissue Ca^{2+} *content.* To determine the intracellular Ca^{2+} at various stages, experiments were terminated and hearts perfused with cold perfusate (0°) containing 100 mM THAM (Tris-[hydroxymethyl]aminomethane) and 220 mM glucose (pH 7.4) for 5 min to wash out the extracellular Ca^{2+} as described previously [15]. This method has been shown to be able to wash out more than 95% of extracellular Ca^{2+} [15]. After the perfusion, the hearts were dried and ashed at 550° for 20 hr. The ash was dissolved in 5 mL of 3 M nitric acid and diluted 10-fold with distilled water. Ca^{2+} was measured at 422.7 nm using a Perkin–Elmer 1100 B atomic absorption spectrophotometer. The Ca^{2+} concentration was expressed as micromoles per gram dry weight.

Assay for creatine kinase. Recirculating perfusates collected at different stages of the experiment were assayed for CK activity by the enzymatic method, as described elsewhere, using a CK assay kit obtained from the Sigma Chemical Co.

Assay for lipid peroxidation. Myocardial lipid peroxidation was determined by measuring tissue thiobarbituric acid (TBA) reactive substances (TBARS) as described previously [16]. LV tissue (50–75 mg) from a similar anatomical location (LV free wall) was homogenized in 2 mL of 15% trichloroacetic acid; then 1 mL of 0.75% TBA and 0.5% sodium acetate was added into the tissue homogenate. The mixture was boiled for 15 min. The red color of the TBA–malondialdehyde complex was measured at 535 nm with a spectrophotometer.

Free radical scavenging activity of dihydropyridines. The abilities of dihydropyridines to scavenge the free radicals were studied by generating superoxide anion (O_2^-) from the reaction of xanthine on xanthine oxidase (XO); hydroxyl radical ($OH^\cdot$) from the reaction of xanthine, XO, $FeCl_3$, and EDTA; and hypohalide radical ($OCl^\cdot$) from sodium hypochlorite; and then monitoring the abilities of the dihydropyridines to inhibit the chemiluminescence response produced from the reaction of free radicals on luminol as described previously [17]. Luminol was added to the free radical generating system at pH 10, and the light generated during the reaction was measured using a luminometer. Quantification

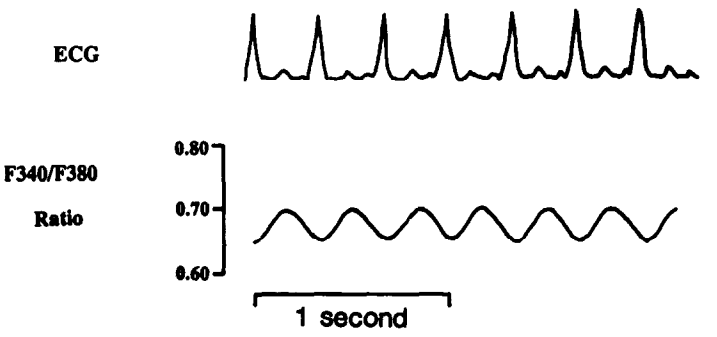


Fig. 1. Representative tracing of intracellular Ca^{2+} transient ($[Ca^{2+}]_i$) obtained from a non-ischemic heart. ECG indicates the electrocardiogram; F_{340}/F_{380} ratio indicates the $[Ca^{2+}]_i$.

of luminol luminescence was based on the reaction of HX + XO producing uric acid + O_2^-, which was taken as 100%. In addition, the abilities of dihydropyridine analogs to scavenge O_2^- was monitored by ferricytochrome *c* reduction [17]. The ability to scavenge OH· radical was monitored using HPLC as described previously [18]. DHPs (dissolved in ethanol and diluted with buffer to the desired concentrations) were added into reaction mixtures. For each free radical species, four to six individual determinations were performed at each given concentration of DHP.

Statistical analysis. All the results are expressed as means ± SEM of six separate experiments in each group. Students *t*-test was used for comparisons between two groups. One-way analysis of variance followed by Bonferroni's *t*-test were performed for the multiple group comparisons by using a statistical analysis software program (Primer, McGraw-Hill, Inc., 1988). $P < 0.05$ was considered to be significant.

RESULTS

Effects of DHPs on intracellular Ca^{2+} *transient* ($[Ca^{2+}]_i$). An intracellular calcium analyzer was used to examine $[Ca^{2+}]_i$ transient during ischemia and reperfusion. This instrument allowed us to monitor the beat-to-beat change of $[Ca^{2+}]_i$ that occurred during each cardiac cycle. Figure 1 shows the typical $[Ca^{2+}]_i$ obtained in a normal rat heart. The F_{340}/F_{380} ratio indicates the cytosolic free Ca^{2+} which changed corresponding to the electrocardiogram. The $[Ca^{2+}]_i$ transient was monitored continuously for both the control and the experimental groups throughout the experiment. The $[Ca^{2+}]_i$ increased immediately after the induction of ischemia (10–15% increase of the F_{340}/F_{380} ratio over baseline); the $[Ca^{2+}]_i$ rose steadily during 30 min of ischemia and peaked at the first few minutes of reperfusion as shown in Fig. 2. The DHP-treated groups also showed a slight rise of $[Ca^{2+}]_i$ during

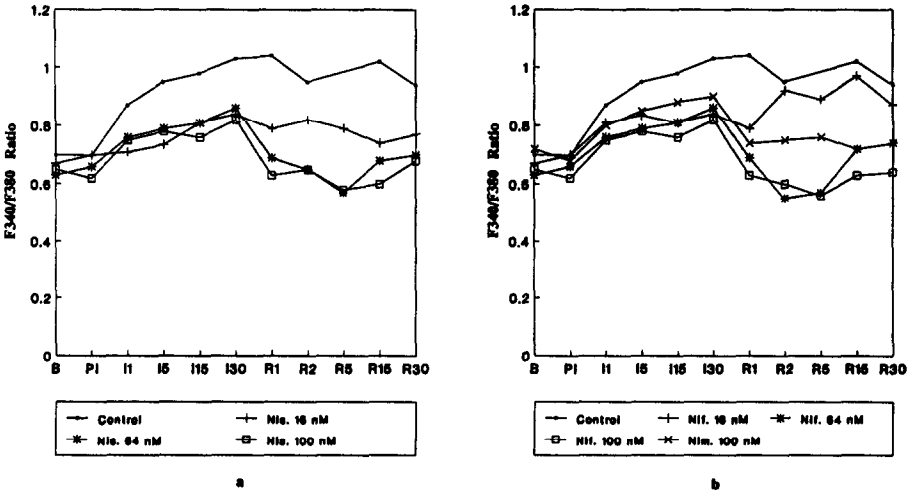


Fig. 2. Effects of DHPs in the changes of $[Ca^{2+}]_i$ (F_{340}/F_{380} ratio) during ischemia and reperfusion. The left panel (a) depicts the effect of nisoldipine; the right panel (b) depicts the effects of nifedipine and nimodipine. B = baseline; PI = pre-ischemia; I1, I5, I15 and I30 indicate the time points (min) during ischemia; R1, R2, R5, R15, and R30 indicate the time points (min) during reperfusion.

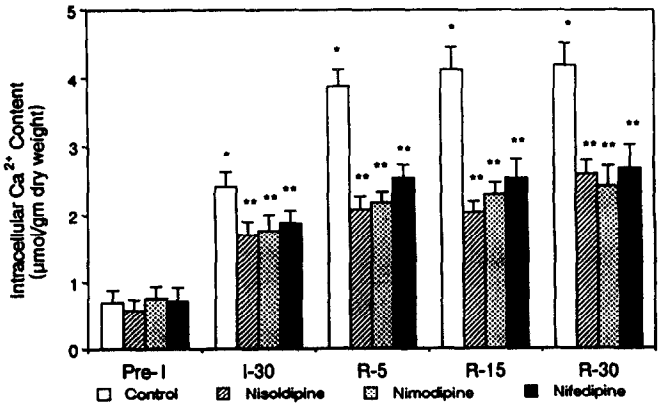


Fig. 3. Change of tissue Ca^{2+} content measured during ischemia and reperfusion. The Ca^{2+} was measured by atomic absorption spectroscopy as described in Materials and Methods. Hearts were treated with a 100 nM concentration of the three DHPs. Results are expressed as means ± SEM of six hearts in each group. Key: (*) $P < 0.05$ compared with Pre-I; and (**) $P < 0.05$ compared with control group.

ischemia, but the amplitude of the rise of $[Ca^{2+}]_i$ was decreased, and the $[Ca^{2+}]_i$ returned to baseline more rapidly in treated groups than in the control group. As shown in Fig. 2, all three DHPs tested at the higher concentration (100 nM) reduced the rise of the $[Ca^{2+}]_i$ transients during ischemia and reperfusion. In the concentration–response study, the effective concentration for nisoldipine, nifedipine and nimodipine appeared to be 16, 64, and 100 nM, respectively. We were unable to quantify the intracellular Ca^{2+} in isolated rat hearts with this technique, and more efforts are needed to establish its validity in quantifying intracellular Ca^{2+}.

Effects of DHPs on tissue Ca^{2+} *concentration.* Myocardial Ca^{2+} content was also measured using atomic absorption spectroscopy. There was no change in tissue Ca^{2+} content up to 15 min of ischemia in any of the groups, but there was a rise in Ca^{2+} concentration after 30 min of ischemia (Fig. 3). This increase was partially, but significantly inhibited by the dihydropyridines. Reperfusion of ischemic myocardium was associated with a further increase in Ca^{2+} concentrations. Again, dihydropyridines were able to inhibit the increase in Ca^{2+} content significantly.

Effects of DHPs on postischemic left ventricular function. During the perfusion with DHPs prior to ischemia, the myocardial contractile function did not change significantly as indicated by the unchanged LV developed pressure and LV dp/dt_{max} compared to baseline values (data not shown). The recoveries of LV developed pressure (Table 1) and LV dp/dt_{max} (Table 2) during reperfusion were significantly improved in nisoldipine (effective concentration 16 nM), nifedipine (effective concentration 64 nM), and nimodipine (100 nM)-treated groups compared with the control group. LV end-diastolic pressure (LVEDP) values were comparable between control and treated groups under baseline conditions; however, LVEDP was increased significantly in control from a baseline value of 9 ± 1.5 to 18 ± 2 mm Hg at the end of 30 min of reperfusion ($P < 0.05$ compared with baseline). The DHP-treated hearts exhibited a less significant rise of LVEDP, when

Table 1. Effects of dihydropyridines on left ventricular developed pressure (LVDP) in isolated rat hearts

DHP concentrations (nM)	LVDP (mm Hg)							
	Control		Nisoldipine		Nifedipine		Nimodipine	
	Base	R30	Base	R30	Base	R30	Base	R30
0	93 ± 7	52 ± 4						
4			96 ± 8	58 ± 5	97 ± 6	56 ± 4	98 ± 7	53 ± 5
16			95 ± 7.5	70 ± 5.3*	95 ± 4	55 ± 3	100 ± 9	57 ± 6
64			94.5 ± 7	72 ± 5.8*	97 ± 5	66 ± 3.8*	96 ± 8	60 ± 5.8
100			95 ± 10	69 ± 5*	97 ± 5	67 ± 4.6*	92 ± 10	66 ± 4.6*

Results are expressed as means ± SEM of six experiments in each group. Base = baseline; R30 = 30 min of reperfusion.

* $P < 0.05$ compared with the R30 control group.

Table 2. Effects of dihydropyridines on LV dp/dt_{max} in isolated rat hearts

DHP concentrations (nM)	LV dp/dt_{max} (mm Hg/sec)							
	Control		Nisoldipine		Nifedipine		Nimodipine	
	Base	R30	Base	R30	Base	R30	Base	R30
0	1430 ± 170	760 ± 60						
4			1450 ± 120	750 ± 80	1460 ± 110	750 ± 86	1460 ± 150	750 ± 66
16			1460 ± 110	960 ± 58*	1470 ± 130	740 ± 72	1440 ± 120	760 ± 69
64			1430 ± 115	1100 ± 100*	1430 ± 150	980 ± 65*	1420 ± 110	740 ± 70
100			1460 ± 130	1000 ± 75*	1460 ± 110	960 ± 62*	1420 ± 150	930 ± 55*

Results are expressed as means ± SEM of six experiments in each group. Base = baseline; R30 = 30 min of reperfusion.
* $P < 0.05$ compared with the R30 control group.

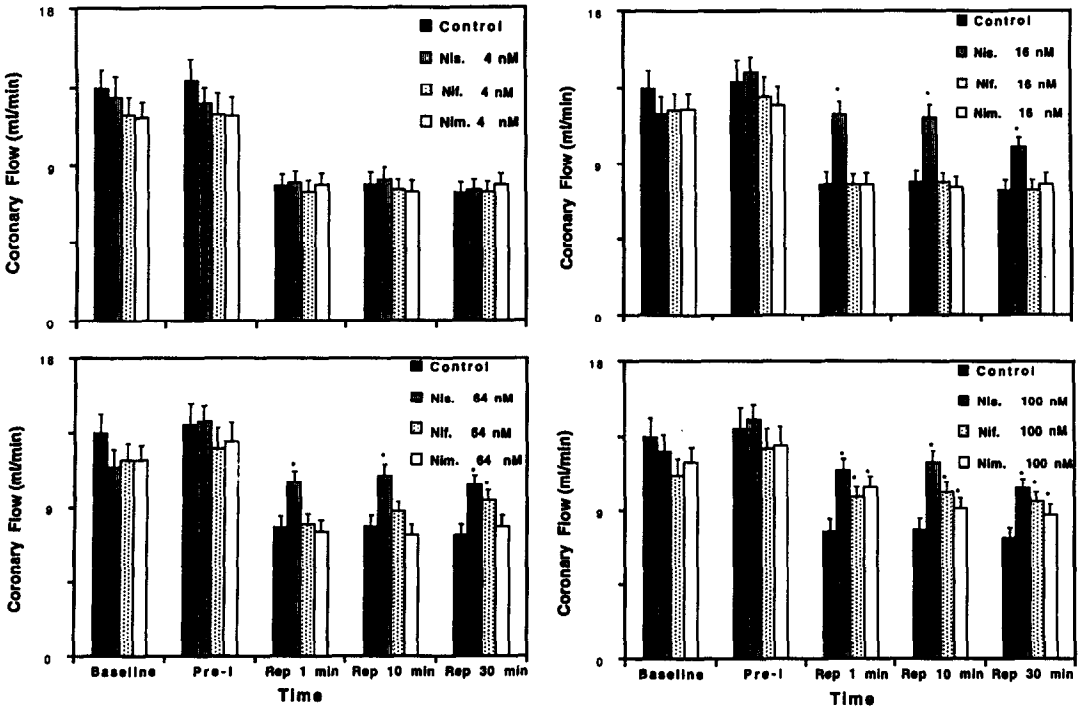


Fig. 4. Effects of concentrations of DHPs on the changes of coronary flow. Pre-I = pre-ischemia, R_1, R_{10}, and R_{30}, indicate 1, 10, and 30 min of reperfusion. Results are expressed as means ± SEM of six hearts in each group. Abbreviations: Nis, nisoldipine; Nim, nimodipine; and Nif, nifedipine. Key: (*) $P < 0.05$ compared with the non-treated group.

higher concentrations (100 nM) of DHP were used; LEVDP values determined at the 30 min time point of reperfusion were 12 ± 0.9, 14 ± 1.1 and 13 ± 1.2 mm Hg for nisoldipine, nifedipine and nimodipine, respectively ($P < 0.05$ compared with control group). However, only nisoldipine was able to decrease the rise of LVEDP (14 ± 1.0 vs 18 ± 1.2 mm Hg, $P < 0.05$, nisoldipine control) significantly at a lower concentration (16 nM).

Effects of DHPs on the recovery of coronary flow. Slight vasodilation was observed during perfusion with DHPs as shown in Fig. 4. The recovery of coronary flow was monitored throughout the reperfusion. When the hearts received the higher concentration of DHPs (100 nM), they all showed significantly better recovery of coronary flow compared with the control group ($P < 0.05$). Nisoldipine was effective in improving the coronary flow even at a concentration of 16 nM. Nifedipine appeared to be more effective than nimodipine in enhancing the recovery of coronary flow (Fig. 4).

Effect of DHPs on myocardial CK release. Myocardial CK release was minimal prior to ischemia in all groups. During reperfusion, CK release was increased significantly as a function of time, but in the treated groups the levels of CK release was

Table 3. Effects of dihydropyridines on myocardial creatine kinase release

	Creatine kinase release (IU/L)									
	Control	Nisoldipine (nM)			Nifedipine (nM)			Nimodipine (nM)		
		16	64	100	16	64	100	16	64	100
Baseline	2.6 ± 0.19	2.6 ± 0.5	2.4 ± 0.3	2.5 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.5 ± 0.3	2.4 ± 0.25	2.5 ± 0.26	2.4 ± 0.3
Pre-ischemia	3.2 ± 0.79	3.2 ± 0.2	3.0 ± 0.2	2.9 ± 0.19	3.2 ± 0.3	3.0 ± 0.28	3.3 ± 0.2	3.1 ± 0.29	2.9 ± 0.15	3.4 ± 0.2
Rep. 1 min	17 ± 3.2	16 ± 1.8	14 ± 1.5	12 ± 0.9*	19 ± 1.4	13 ± 1.2	19 ± 1.1	19 ± 1.5	16.8 ± 1.2	11 ± 0.8*
Rep. 10 min	28 ± 1.6	21 ± 1.5*	22 ± 1.3*	19 ± 1.2*	27 ± 2.4	21 ± 1.8*	22 ± 1.5*	27 ± 2.4	25.6 ± 2.5	18 ± 1.7*
Rep. 30 min	36 ± 2.5	29 ± 1.5	27 ± 1.9*	26 ± 1.8*	37 ± 3.9	31 ± 2.6*	30 ± 2.3*	38 ± 2.3	34.5 ± 3.1	27 ± 1.8*

Results are expressed as means ± SEM of six separate experiments in each group. Rep. 1, Rep. 10, and Rep. 30 min indicate different times of reperfusion.
* $P < 0.05$ compared with control groups.

decreased significantly compared with the control group (Table 3). Again, a difference in concentration–response existed among the three DHPs tested in this study; nisoldipine was effective at a relatively lower dose.

Effect of DHPs on myocardial lipid peroxidation. Myocardial lipid peroxidation was determined by measuring the formation of TBARS in the tissue obtained at the end of reperfusion. Nisoldipine, nifedipine and nimodipine all decreased the TBARS in the myocardium when a 100 nM concentration of each drug was used (Fig. 5), indicating that lipid peroxidation could be decreased by dihydropyridines. At lower concentration (64 nM), only nisoldipine and nifedipine decreased TBARS (Fig. 5).

Free radical scavenging activity of DHPs. None of the dihydropyridines demonstrated direct free radical scavenging actions when examined by their abilities to inhibit the chemiluminescence generated by a O_2^-, $^{\cdot}OH$, or $OCl^{\cdot}$ generating system. They were also equally ineffective in scavenging O_2^- when examined by their abilities to inhibit ferricytochrome *c* reduction. None of the dihydropyridines were able to scavenge the $OH^{\cdot}$ when examined by HPLC (data not shown).

DISCUSSION

Dihydropyridine Ca^{2+} channel blockers function by inhibiting the myocardial slow inward Ca^{2+} current (I_{Ca}) by interacting with the receptor located on the Ca^{2+} channel [19]. These drugs, in general, possess potent arteriolar vasodilatory properties [20]. The most common dihydropyridine, nifedipine, is widely used to treat angina and hypertension. Among other dihydropyridine drugs, nisoldipine has been shown to be more potent than nifedipine in pharmacological studies [12, 13], and it has also been used clinically for the treatment of cardiovascular disease, whereas nimodipine is used generally as a cerebral vasodilator [21]. However, recent studies have demonstrated the effectiveness of these drugs against myocardial ischemic injury [22, 23]. Vasodilatory action and Ca^{2+} antagonism properties, either alone or in combination may have contributed to myocardial preservation. The results of this study indicated that the dihydropyridines can reduce myocardial ischemic and reperfusion injury by reducing the rise of intracellular Ca^{2+} during ischemia and reperfusion and by increasing coronary blood flow during the reperfusion of ischemic myocardium.

There are numerous studies which demonstrate that myocardial ischemia and reperfusion are associated with an increase in the intracellular Ca^{2+} content [24–26]. However, the exact mechanism of intracellular Ca^{2+} overloading is not known. Current knowledge suggests that a significant route of Ca^{2+} influx during post-ischemic reperfusion is via the Na^+/Ca^{2+} exchanger mechanism [27]. During ischemia, tissue levels of ATP fall, resulting in insufficient energy to operate the Na^+-K^+-ATPase pump [28]. In addition, accumulation of protons results in a reduction of intracellular pH [29]. Na^+ gains access in exchange for H^+; then cytosolic Na^+ is exchanged for extracellular Ca^{2+}. In addition to this major route, Ca^{2+} can gain access into the cell

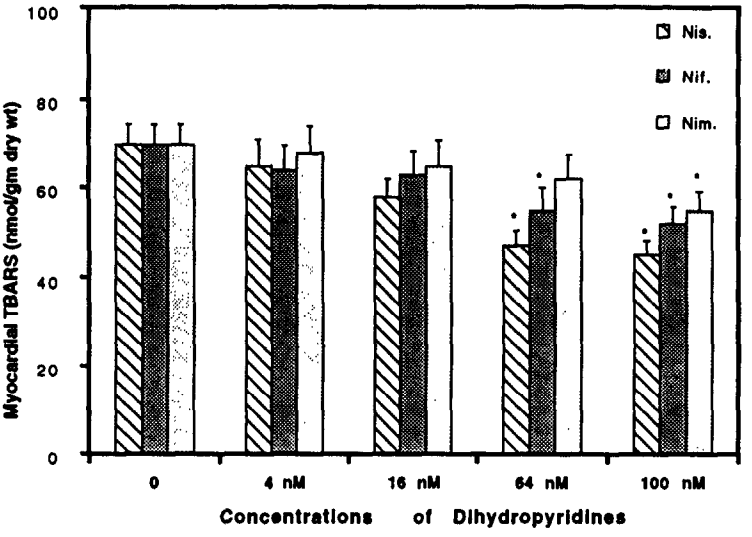
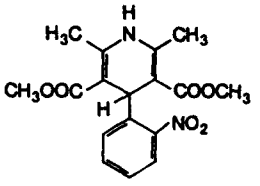


Fig. 5. Effects of DHPs on myocardial TBA reactive substances (TBARS). Biopsies were taken from LV at the end of 30 min of reperfusion. Results are expressed as means ± SEM of six hearts in each group. Abbreviations: Nis, nisoldipine; Nif, nifedipine; and Nim, nimodipine. Key: (*)$P < 0.05$ compared with the non-treated group.

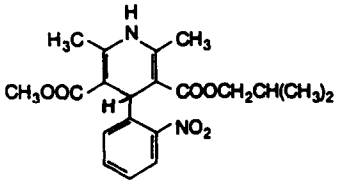
by other routes including the Ca^{2+} slow channel and as an exchange for K^+ [30]. It is generally believed that during reversible ischemia and during reperfusion, when the sarcolemma is intact, Ca^{2+} gains access into the cell via the receptor and voltage-regulated Ca^{2+} channels [6]. Our study also supports this concept, because all of the Ca^{2+} slow channel blocking dihydropyridine drugs reduced the $[Ca^{2+}]_i$ transients and myocardial Ca^{2+} contents during ischemia and reperfusion. In this study, we used two independent techniques to examine the change of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ transient increased immediately after the onset of ischemia and then rose gradually during ischemia, as shown in Fig. 2. This rise of $[Ca^{2+}]_i$ during early ischemia was decreased partially by DHPs, indicating that there are also some other mechanisms for the rise of cytosolic free Ca^{2+}. It has been suggested that the energy-dependent uptake of cytosolic free Ca^{2+} by sarcoplasmic reticulum may be impaired as a result of depletion of ATP and development of intracellular acidosis caused by cardiac ischemia [31]. Significant reduction of uptake of Ca^{2+} by sarcoplasmic reticulum was found after 7.5 min of ischemia [31]. Whether the increase of Ca^{2+} during the first few minutes of ischemia can be explained by sarcoplasmic reticulum dysfunction is still unknown. Mohabir *et al.* [32] examined the effects of transient ischemia on $[Ca^{2+}]_i$ and intracellular pH in isolated rabbit hearts using indo-1 and BCECF (a pH probe) as probes. They found that the significant rise of Ca^{2+} during the first 2 min of ischemia, and the decrease of intracellular pH in the first few minutes of ischemia could not explain the rise of $[Ca^{2+}]_i$. These authors speculated that this early rise may be due to the release of endothelin. However, the mechanisms responsible for the rise of Ca^{2+} during early ischemia need to be elucidated.

The dihydropyridines used in our study increased coronary flow during the reperfusion of ischemic heart in a concentration-dependent manner. It seems likely that the vasodilatory effects of these drugs may improve coronary perfusion, thus reducing the post-ischemic coronary perfusion dysfunction. Several pharmacological studies demonstrated the difference in potency between nisoldipine and nifedipine. Experimental coronary spasms are prevented at a lower dose by nisoldipine compared with nifedipine [33]. Aortic strips or portal veins contracted by an increase in the calcium concentration in the bath solution are relaxed by low concentrations (1.1×10^{-9} and 7.6×10^{-10} mol/L) of nisoldipine [12]. The drug exerts a similar strong action on isolated rabbit mesenteric arteries [34]. The duration of the inhibitory effect of nisoldipine was investigated in isolated potassium-depolarized aortic strips [35]. In this preparation the duration of action of nisoldipine was approximately twice as long as that of nifedipine. Nisoldipine and nifedipine possess higher cardioselectivity than nimodipine, whereas nimodipine is more potent in antagonizing the calcium channel in the cerebrovascular system due to its high lipophilicity [21]. In the present study, we did not observe any significant effect of 4 nM nisoldipine in improving the recovery of coronary flow, but 16 nM nisoldipine increased the recovery of coronary flow significantly and it was more potent in its ability to reduce myocardial ischemic/reperfusion injury than nifedipine. The present results also indicate that nifedipine is more potent than nimodipine in reducing myocardial injury.

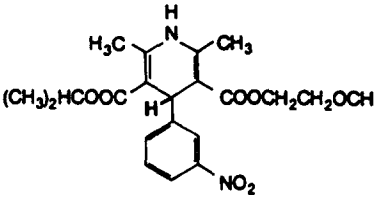
One interesting observation in this study is probably the reduced contents of lipid peroxidation products by the dihydropyridines at relatively higher concentrations. Our results indicated that none of the dihydropyridine compounds possessed direct free radical scavenging properties, yet they were



Nifedipine



Nisoldipine



Nimodipine

Fig. 6. Structural skeletons of nifedipine, nisoldipine, and nimodipine.

able to reduce the extent of lipid peroxidation during the reperfusion of ischemic heart. A recent study showed that nifedipine could inhibit lipid peroxidation of liver microsomal membrane exposed to free radicals, an observation independent of its Ca^{2+}-inhibiting properties [36]. The authors assumed that nifedipine probably reduced the lipid peroxidation by its membrane-stabilizing properties [36]. It has been speculated that the antioxidative mechanism of DHPs such as nifedipine might be similar to a chain-breaking reaction at the level of membrane phospholipids [37]. Recently Mak *et al.* [38] demonstrated that antioxidant effects of calcium channel blockers against free radical injury in endothelial cells were not due to the effects of these agents in scavenging the free radicals generated in the aqueous phase; their results suggested that the protective mechanisms were mediated by the lipid antiperoxidative activities of these compounds and were associated with the preservation of cellular glutathione content [38]. Our results also support their findings. DHPs examined in this study did not show any scavenging effects against superoxide anion or hydroxyl radical under *in vitro* conditions. However, these DHPs may incorporate into cardiac membranes due to their lipophilicity, thus decreasing the membrane lipid peroxidation. These abilities to prevent peroxidation of DHPs would certainly contribute towards the reduction of myocardial reperfusion injury. These dihydropyridines have identical structural skeletons (Fig. 6). They differ mostly in terms of the position of the $-NO_2$ substituent on the benzene ring and the bulkiness of the alkyl substituent of the carbonyl ester groups located in the 3- and 5-positions of the pyridine moiety. Bulkiness of the alkyl substituent increases in the order: nifedipine < nisoldipine < nimodipine. In the cases of nifedipine and nisoldipine there is steric crowding following interactions between the ester group at the 3-position of the pyridine ring and the $-NO_2$ substituent of the benzene ring. Bulkiness of the alkyl substituent on the ester group at the 3-position in nisoldipine is much greater than that of nifedipine, hence steric interaction is more likely to occur. These interactions are not at all present in nimodipine. It is obvious that the major reaction site is located on the nitrogen lone pair on the pyridine ring. The electron withdrawing effect is more prominent when the $-NO_2$ substituent is in the 2-position. An electron withdrawal effect is present in nimodipine to some extent due to the methoxy group at the 3-position. It seems that an optimum steric interaction between the nitro group and the ester group may be instrumental in their abilities to reduce the susceptibility of membranes to free radical attack.

In summary, we have shown that three dihydropyridines, nifedipine, nisoldipine, and nimodipine, are able to attenuate myocardial reperfusion injury in a concentration-dependent fashion. These drugs demonstrated several different properties: calcium antagonism, vasodilatory action, and antioxidative properties. Any one or all of these functions may be instrumental in their abilities to reduce the reperfusion injury as observed in our study.

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