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Attenuation of increased myocardial ischaemia-reperfusion injury conferred by hypercholesterolaemia through pharmacological inhibition of the caspase-1 cascade

[1]Tzung-Dau Wang, [2]Wen-Jone Chen, [1]Tzan-Jr Mau, [1]Jong-Wei Lin, [3]Wan-Wan Lin & *,[1]Yuan-Teh Lee

[1]Division of Cardiology, Department of Internal Medicine, National Taiwan University Hospital, Taipei, 100, Taiwan, Republic of China; [2]Department of Emergency Medicine, National Taiwan University Hospital, Taipei, 100, Taiwan, Republic of China and [3]Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, 100, Taiwan, Republic of China

1 Hypercholesterolaemia has been shown to be associated with greater myocardial ischaemia-reperfusion injury, in which apoptosis and inflammation-mediated necrosis both play a key role.

2 Caspase-1 is involved in the activation of both apoptosis and inflammation, through the intermediate of interleukin-1β (IL-1β). We herein examined whether pharmacological inhibition of the caspase-1 cascade, using Ac-Tyr-Val-Ala-Asp-CH_2Cl (Ac-YVAD.cmk), after myocardial ischaemia have greater protective effects on myocardial ischaemia-reperfusion injury in diet-induced hypercholesterolaemic rabbits.

3 Male rabbits fed with standard chow or chow supplemented with 0.5% cholesterol and 10% coconut oil for 8 weeks were subjected to 30 min of left circumflex artery occlusion followed by 4 h of reperfusion. An intravenous bolus of Ac-YVAD.cmk (1.6 mg kg^{-1}) or vehicle was given 20 min after coronary occlusion.

4 Postischaemic administration of Ac-YVAD.cmk markedly decreased infarct size from $26\pm3\%$ to $12\pm2\%$ in normally fed rabbits ($P=0.005$) and from $41\pm6\%$ to $14\pm2\%$ in cholesterol-fed rabbits ($P<0.001$).

5 In the ischaemic non-necrotic area, treatment with Ac-YVAD.cmk markedly reduced the percentage of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)-positive cardiomyocytes from $15.5\pm0.8\%$ to $2.2\pm0.1\%$ in normally fed rabbits ($P<0.001$) and from $39.0\pm2.3\%$ to $2.2\pm0.1\%$ in cholesterol-fed rabbits ($P<0.001$).

6 Ac-YVAD.cmk treatment resulted in a reduction not only of IL-1β and caspase-1, but also of caspase-3 in the ischaemic myocardium in both normally fed and cholesterol-fed rabbits.

7 No differences in infarct size, the percentage of TUNEL-positive cardiomyocytes, IL-1β levels or activity of caspase-1 and caspase-3 were observed between Ac-YVAD.cmk-treated normally fed and cholesterol-fed rabbits.

8 This study demonstrates that injection of a selective caspase-1 inhibitor after myocardial ischaemia markedly reduced the detrimental effect conferred by hypercholesterolaemia on myocardial ischaemia-reperfusion injury by attenuating both necrotic as well as apoptotic cell death pathways through inhibition of IL-1β production and activation of caspase-1 and caspase-3. *British Journal of Pharmacology* (2003) **138,** 291–300. doi:10.1038/sj.bjp.0705098

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Abbreviations: Ac-YVAD.cmk, Ac-Tyr-Val-Ala-Asp-CH_2Cl; HR, heart rate; IL-1β, interleukin-1β; MABP, mean arterial blood pressure; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling

Introduction

Prompt reperfusion of jeopardized myocardium by angioplasty or thrombolytic therapy is now the standard of care in patients with acute myocardial infarction. However, several recent studies have shown that hypercholesterolaemic individuals have higher mortality rate and worse left ventricular systolic function following myocardial infarction than normocholesterolaemic individuals in the current reperfusion era (Wang *et al*., 1998; Ballantyne *et al*., 2001). This finding suggests that hypercholesterolaemia may adversely influence the evolution of myocardial infarction even after the establishment of a patent infarct-related artery.

Among the factors contributing to delayed injury progression in reperfused myocardium, cardiomyocyte apoptosis and inflammation-mediated necrosis both play a key role (Gottlieb *et al*., 1994; Fliss & Gattinger, 1996; Olivetti *et al*., 1996; Yaoita *et al*., 2000). We have recently demonstrated that the magnitude of cardiomyocyte apoptosis following ischaemia and reperfusion doubled in diet-induced hypercholesterolaemic rabbits (Wang *et al*., 2002). Moreover, several

*Author for correspondence at: Division of Cardiology, Department of Internal Medicine, National Taiwan University Hospital, 7, Chung-Shan South Road, Taipei, 100, Taiwan, Republic of China; E-mail: ytlee@ha.mc.ntu.edu.tw

previous studies have demonstrated that hypercholesterolaemia is associated with increased inflammatory cell infiltration and massive generation of oxygen-derived free radicals in ischaemic-reperfused myocardium (Ohara *et al*., 1993; Hoshida *et al*., 1999). In keeping with these observations, most previous experiments have shown that hypercholesterolaemia results in larger infarct size in hearts exposed to ischaemia and reperfusion (Golino *et al*., 1987; Tilton *et al*., 1987; Osborne *et al*., 1989). Nevertheless, some recent studies showed that prolonged exposure to hypercholesterolaemia (over 5 weeks) did not harm or even protected the heart from reperfusion injury (Ferdinandy *et al*., 1997; Le Grand *et al*., 1995; Girod *et al*., 1999; Kremastinos *et al*., 2000). Hence, the effects of prolonged hypercholesterolaemia on reperfusion injury after myocardial ischaemia still deserve further investigation.

Mechanistically, apoptotic cell death is essentially mediated by a family of aspartate-specific cysteine proteases known as caspases that include at least 14 members (Yaoita *et al*., 2000). Caspase-1, the first identified member of the caspase family, is responsible for the activation of executioner caspases involved in apoptosis progression in a variety of experimental paradigms (Denner, 1999). Furthermore, it modulates the inflammatory reaction by processing the maturation of interleukin-1β (IL-1β). Caspase-1 hence has the peculiarity of being involved in the activation of both apoptosis and inflammation (Kuida *et al*., 1995). Additionally, recent studies have shown that oxidized low-density lipoprotein, a key substance mediating hypercholesterolaemia-related atherogenesis, induces endothelial apoptosis by activating caspase-1, through the intermediate of free radicals (Harada-Shiba *et al*., 1998; Kotamraju *et al*., 2001). Based on these background researches, we speculated that pharmacological inhibition of the caspase-1 cascade might have greater protective effects on myocardial ischaemia-reperfusion injury in the context of hypercholesterolaemia.

In this study, we used the caspase-1-like protease inhibitor Ac-Tyr-Val-Ala-Asp-CH_2Cl (Ac-YVAD.cmk) (Holly *et al*., 1999; Rabuffetti *et al*., 2000) and administered it after having produced myocardial ischaemia in a diet-induced hypercholesterolaemic rabbit model. We herein confirmed that prolonged hypercholesterolaemia (8 weeks) is associated with greater myocardial ischaemia-reperfusion injury. Furthermore, our report is the first to demonstrate that pharmacological inhibition of the caspase-1 cascade markedly reduced the detrimental effect conferred by hypercholesterolaemia on myocardial ischaemia-reperfusion injury, achieved by interfering with both apoptotic and inflammatory mechanisms.

Methods

Animals and diet

Adult male New Zealand white rabbits (2.4–2.8 kg) were housed in individual cages, in environmentally controlled rooms (12 h light-dark cycle, humidity $50\pm5\%$ and $20\pm1^{\circ}C$). Sixty rabbits were randomly assigned to two different dietary groups: animals in the control group ($n=30$) were fed standard rabbit pellets (Purina 5321, St Louis, MO, U.S.A.), whereas those in the cholesterol-fed group ($n=30$) received a diet enriched with 0.5% cholesterol and 10% coconut oil added to the standard Purina rabbit pellets for a total of 8 weeks. Rabbits were carefully scheduled to start their special diets so that they were mature on the same day 8 weeks later. At the end of the 8-week feeding period, the rabbits were studied. The animals had free access to water. Blood samples were taken for determination of plasma levels of cholesterol and triglycerides just before the administration of the diet and at the end of the 8-week feeding period. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The experimental protocol was approved by the Institutional Animal Care and Use Committee.

Surgical procedure and treatments

After a 12-hour fasting, the rabbits were anaesthetized with pentobarbital (25 mg kg^{-1} i.v.) and anaesthesia maintained during the experiment by intravenous injection of small amounts of pentobarbital (5 mg kg^{-1}), sufficient to abolish the corneal reflex. An intratracheal tube was inserted through a midline incision, and all rabbits were given intermittent positive-pressure ventilation *via* a Harvard small animal respirator (Harvard Apparatus, Holliston, MA, U.S.A.). The respirator was adjusted to maintain arterial blood gases within the physiological range.

The standard limb leads of the electrocardiogram were monitored during the experiment. A polyethylene catheter was inserted through the right femoral artery and positioned in the abdominal aorta for blood gas and arterial pressure monitoring (Gould Instruments, Essex, U.K.). A midline sternotomy was performed, and the heart was exposed after the pericardium was incised. A 5-0 silk suture on a small curved needle was passed through the myocardium beneath the major marginal branch of the left circumflex coronary artery located on the dorsal surface of the heart, 10–12 mm from its origin. A reversible tie was subsequently made and loosely placed on the myocardial surface. After a 20-min stabilization period after thoracotomy, myocardial ischaemia was initiated by complete ligation of the marginal coronary artery. Myocardial ischaemia was confirmed by ST-segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. The ligature was released after 30 min of ischaemia and the ischaemic myocardium was reperfused for 4 h. Reperfusion was confirmed by myocardial blush over the risk area after the ligature was released. We eliminated the rabbits that did not complete the experiment from analyses.

To assess the impact of caspase-1 cascade inhibition, rabbits of both normally fed and cholesterol-fed groups were given either an intravenous bolus of Ac-YVAD.cmk (BACHEM, 1.6 mg kg^{-1} in DMSO) or vehicle (DMSO, 80 μg kg^{-1}) 20 min after coronary occlusion. One mg of Ac-YVAD.cmk was dissolved in 50 μl of DMSO.

Determination of area at risk and infarct size

At the end of the reperfusion period, the marginal coronary artery was again occluded through ligation of the tie that remained at the site of the previous occlusion. Immediately after the ligation, 5 ml of 1% Evans blue dye (Sigma, St. Louis, MO, U.S.A.) was injected directly into the left atrial cavity to delineate the area of the left ventricular myocardium

perfused by the patent coronary arteries. The area-at-risk was thus determined by negative staining. The heart was then rapidly removed and placed in ice-cold 0.9% saline, and the atria, right ventricle, and great vessels were removed. The left ventricle was sliced into sections 2-mm thick parallel to the atrioventricular groove. The unstained portion of the myocardium (i.e., the area-at-risk) was separated from the stained portion (i.e., the non-ischemic area). The unstained portion was subsequently sliced into 1-mm thick sections and counterstained with 10 ml of 1% solution of the 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, U.S.A.) in 20 mM phosphate-buffered saline, pH 7.4 at 37°C for 15 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium, which did not stain, was separated from the stained portion (i.e., ischaemic non-necrotic area). All three portions of the left ventricular myocardium (i.e., non-ischaemic, ischaemic non-necrotic, and ischaemic necrotic) were weighed individually. The individual portions of the myocardium were further sectioned into 1×1 mm transmural myocardial columns and either fixed in 10% buffered formalin for *in situ* nick end labelling or rapidly frozen in liquid nitrogen and stored at -80°C for DNA and protein isolation. The whole procedure took approximately 25 min to complete. Because differences in this additional time may affect comparability of the results of apoptosis analyses, we carefully monitored the whole procedure to ensure that the time taken for processing both ischaemic and non-ischaemic myocardium were the same.

In situ *nick end labelling*

The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) protocol was performed with the use of a FragEL DNA fragment detection kit (Oncogene, Cambridge, MA, U.S.A.) according to the manufacturer's instructions with minor modifications. Briefly, the fixed transmural ventricular slices were embedded in paraffin and 4-μm thick sections were deparaffinized by washing in xylene and a descending ethanol series. Subsequently, the tissue sections were stripped of proteins through incubation with 20 μg ml^{-1} proteinase K for 15 min at room temperature. The slides were incubated with 3% hydrogen peroxide for 5 min to allow inactivation of endogenous peroxidase and then incubated for 90 min at 37°C with terminal deoxynucleotidyl transferase and biotinylated dUTP to label the 3′-OH ends of DNA. After the end labelling, the slides were coated with streptavidin-conjugated peroxidase for 30 min at room temperature and visualized with the use of chromogen 3,3′-diaminobenzidine and hydrogen peroxide. Counterstaining was performed with methyl green. Using this method, each cardiomyocyte could be defined, and TUNEL-positive or -negative nuclei were stained dark brown or light green, respectively, under light microscopy. When the TUNEL method was performed, positive controls were always included. As a positive control, sections of heart tissue were exposed to DNase I for 20 min before nick end labelling. The numbers of cardiomyocyte nuclei stained were easily identified (data not shown).

Cardiomyocytes from six separate sections that were picked randomly from three of the transmural slices of both non-ischaemic and ischaemic non-necrotic portions of the left ventricular myocardium were analysed per animal. In each section, cardiomyocytes with counterstained nuclei were counted in 20 random high-power fields ($\times 400$) from the endocardial to epicardial portion and the index of apoptosis was determined (i.e., number of apoptotic myocytes/total number of myocytes $\times 100$). TUNEL-positive cardiomyocytes were carefully distinguished from TUNEL-positive noncardiomyocytes, such as macrophages. This evaluation was carried out independently by two persons who were unaware of the experimental protocol.

DNA laddering

Frozen tissue samples (20–30 mg each) were minced in 600 μl of lysis buffer (Puregene DNA Isolation Kit, Minneapolis, MN, U.S.A.) and quickly homogenized on ice. The tissue was digested with 150 μg ml^{-1} of proteinase K at 55°C for 16 h and incubated with RNase A at 37°C for 2 h. After incubation, tissues were precipitated and centrifuged at 14,000 r.p.m. for 10 min. The DNA in supernatants was extracted with phenol/chloroform and precipitated with isopropanol. After centrifugation at 14,000 r.p.m. for 30 min, the resulting DNA pellets were washed with 70% ethanol and dissolved in TE buffer (10 mM Tris and 1 mM EDTA). The concentration and purity of DNA were determined by the measurement of the optical density at 260 nM and the ratio of optical density at 260 nM to that at 280 nM.

To amplify the detection of DNA laddering, we employed a terminal deoxynucleotidyl transferase labelling reaction prior to electrophoretic analysis. Briefly, one μg of isolated DNA was added into a reaction mixture containing 60 EU ml^{-1} terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and 6 μM biotinylated dUTP (Boehringer Mannheim, Mannheim, Germany). After 15 min incubation at 37°C, the biotin-labelled DNA was precipitated with ethanol, resuspended in TE buffer, and separated by electrophoresis on a 1.8% agarose gel. The electrophretically separated DNA was subsequently transferred onto a nylon membrane (Millipore, Bedford, MA, U.S.A.) and detected by the Biotin Detection Kit (KPL Laboratory, Gaithersburg, MD, U.S.A.) using a chemiluminescent substrate and exposure on an X-ray film.

Caspase activity assay

In a separate series of experiments, both normally fed and cholesterol-fed rabbits were subjected to 30-min ischaemia followed by 4-h reperfusion with and without Ac-YVAD.cmk. After rabbits were euthanized by pentobarbital overdose, the hearts were excised and the ischaemic and non-ischaemic portions of the left ventricular myocardium were separately dissected. Tissues were homogenized by a polytron homogenizer in ice-cold lysis buffer (2% β-mercaptoethanol, 10 mM Tris, pH 7.4, 140 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} aprotinin, 1% NP-40, and 0.5% deoxycholic acid) and centrifuged at 14,000 r.p.m. for 30 min. Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Homogenates (100 μg of protein) were applied for the measurement of caspase activity by CaspACE assay system (Promega, Madison, WI, U.S.A.) according to the instruction. Briefly, the fluorogenic substrates for caspase-1 and caspase-3 are labelled with

fluorochrome 7-amino-4-methyl coumarin (AMC). The substrates produce a blue fluorescence that can be detected by exposure to UV light at 360 nM. AMC is released from the substrates upon cleavage by caspase-1 or caspase-3. Free AMC produces a yellow-green fluorescence that is measured by a fluorometer at 460 nM. Fluorescent units were converted to pmol AMC using a standard curve generated with free AMC. The values for each sample were then normalized by the value of control (non-ischaemic myocardium of vehicle-treated normally fed rabbits).

Interleukin-1β determination

Mature IL-1β quantification was performed by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.). The myocardium was homogenized by a polytron homogenizer and centrifuged at 14,000 r.p.m. for 30 min. Protein content was determined by the method of Lowry, using bovine serum albumin as the standard. The assay was performed following the manufacturer's instruction. For quantitation, reference curves, obtained using increasing concentration of recombinant mouse IL-1β, were done in parallel. IL-1β levels were measured as the difference (Δ pg ml^{-1} mg of protein^{-1}) between levels in the ischaemic myocardium and that in the non-ischaemic myocardium.

Statistics

All data are given as mean$\pm$s.e.m. Statistical comparisons between means were made by two-way ANOVA followed by *post-hoc* analyses using the Duncan New Multiple Range Test. A value of $P<0.05$ was considered statistically significant.

Results

Characteristics and mortality

There were no significant differences in body weight or plasma lipid concentrations among the groups at the beginning of the 8-week feeding period (Table 1). After the 8-week feeding period, all rabbits exhibited a similar weight gain, whereas plasma total cholesterol and triglyceride levels were markedly increased in cholesterol-fed rabbits than in normally fed rabbits. There were no significant differences in plasma lipid concentrations between the Ac-YVAD.cmk-treated and vehicle-treated groups of cholesterol-fed rabbits or between the groups of normally fed rabbits. Four rabbits died of ventricular fibrillation (three during reperfusion and one during coronary occlusion) in the vehicle-treated group of cholesterol-fed rabbits, whereas only one rabbit died during reperfusion in the other groups. However, differences in mortality rate among the groups did not reach statistical significance ($P=0.15$), which might be limited by the small sample size.

Haemodynamic data

Mean arterial blood pressure was lower in cholesterol-fed rabbits than in normally fed rabbits at baseline, though not statistically significant (Table 2). There were no significant differences in heart rate and mean arterial blood pressure among the groups during ischaemia and reperfusion.

Myocardial infarct size after ischaemia and reperfusion

There was no significant difference in area-at-risk as a per cent of total left ventricular mass, indicating a comparable degree of jeopardy had occurred among the groups (Figure 1). However, the infarct size, expressed as a per cent of area-at-risk, was significantly greater in the vehicle-treated cholesterol-fed rabbits than in normally fed rabbits ($41\pm6\%$ *vs* $26\pm3\%$, $P=0.003$). The infarct size in the Ac-YVAD.cmk-treated rabbits fed a normal diet was significantly smaller than that in the vehicle-treated rabbits fed a normal diet ($12\pm2\%$ *vs* $26\pm3\%$, $P=0.005$). Likewise, the infarct size in the Ac-YVAD.cmk-treated cholesterol-fed rabbits was significantly smaller than that in the vehicle-treated cholesterol-fed rabbits ($14\pm2\%$ *vs* $41\pm6\%$, $P<0.001$). It is noteworthy that the infarct size in the Ac-YVAD.cmk-treated cholesterol-fed rabbits did not differ significantly from that in the Ac-YVAD.cmk-treated normally fed rabbits. This finding indicates that administration of Ac-YVAD.cmk markedly attenuated the detrimental effect conferred by hypercholesterolaemia on the development of myocardial injury following ischemia and reperfusion. There were no obstructive atherosclerotic lesions in the coronary arteries in tissue sections of both normally fed and cholesterol-fed rabbits (data not shown).

In situ *detection of TUNEL positive cells*

Heart tissue from the non-ischaemic area in all four groups exhibited similar but very low levels of staining for TUNEL ($0.03\pm0.01\%$ in each group). In contrast, significant numbers of cardiomyocyte nuclei in the ischaemic non-necrotic (peri-necrotic) area were stained positively for TUNEL in both vehicle-treated normally fed and cholesterol-fed groups (Figure 2A,B, respectively). The percentage of TUNEL-positive cardiomyocytes in the ischaemic non-necrotic area in vehicle-treated cholesterol-fed rabbits was significantly greater than that in vehicle-treated normally fed rabbits ($39.0\pm2.3\%$ vs. $15.5\pm0.8\%$; $P<0.001$) (Figure 3). The positive TUNEL staining was primarily confined to cardiomyocytes, which could be easily distinguished from other non-myocytes by their morphology. Furthermore, apoptotic myocytes were localized to a greater degree in the subendocardial area and were individually dispersed among otherwise normal cardiomyocytes. Ac-YVAD.cmk administered after ischaemia significantly reduced the percentage of TUNEL-positive cardiomyocytes in the ischaemic non-necrotic area in both normally fed and cholesterol-fed rabbits ($2.2\pm0.1\%$ and $2.3\pm0.1\%$, respectively) (Figure 2C,D, respectively), consistent with disappearance of DNA laddering in these two groups (see below). There was no significant difference in the percentage of TUNEL-positive cardiomyocytes in the ischaemic non-necrotic area between normally fed and cholesterol-fed groups. Because DNA degradation occurred nonspecifically in necrotic myocardium and this might also be stained by TUNEL, the extent of cardiomyocyte apoptosis in the necrotic area was thus not assessed.

Table 1 Characteristics of experimental groups

	Normally fed		*Cholesterol-fed*	
	Vehicle-treated (n = 14)	*Ac-YVAD.cmk-treated* (n = 14)	*Vehicle-treated* (n = 10)	*Ac-YVAD.cmk-treated* (n = 14)
Baseline				
Total cholesterol ($mmol^{-1}$)	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
Triglyceride ($mmol^{-1}$)	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Body weight (kg)	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
After 8-week feeding period				
Total cholesterol ($mmol^{-1}$)	1.6 ± 0.1	1.7 ± 0.1	35.6 ± 0.9*	35.4 ± 0.8*
Triglyceride ($mmol^{-1}$)	0.5 ± 0.1	0.5 ± 0.1	2.1 ± 0.1*	2.2 ± 0.1*
Body weight (kg)	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.0 ± 0.1

In the cholesterol-fed groups, laboratory chow was supplemented with 0.5% cholesterol and 10% coconut oil. *$P < 0.001$ vs the vehicle-treated normally fed group.

Table 2 Haemodynamic data

	Normally fed				*Cholesterol-fed*			
	Vehicle-treated (n = 14)		*Ac-YVAD.cmk-treated* (n = 14)		*Vehicle-treated* (n = 10)		*Ac-YVAD.cmk-treated* (n = 14)	
	HR (bpm)	*MABP* (mmHg)	*HR* (bpm)	*MABP* (mmHg)	*HR* (bpm)	*MABP* (mmHg)	*HR* (bpm)	*MABP* (mmHg)
Baseline	251 ± 10	90 ± 6	265 ± 11	90 ± 4	242 ± 8	79 ± 4	242 ± 6	81 ± 3
30 min Ischaemia	264 ± 10	62 ± 5	250 ± 10	60 ± 6	250 ± 9	55 ± 3	240 ± 6	58 ± 3
120 min Reperfusion	252 ± 11	61 ± 3	247 ± 9	61 ± 3	240 ± 9	56 ± 3	245 ± 9	59 ± 2
240 min Reperfusion	265 ± 11	60 ± 3	253 ± 9	62 ± 3	250 ± 11	57 ± 3	255 ± 6	61 ± 3

HR, heart rate; MABP, mean arterial blood pressure.

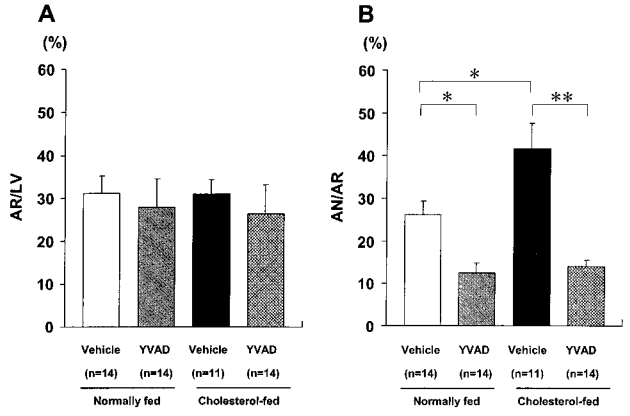

Figure 1 Tissue wet weights of area-at-risk (AR) as a per cent of the total left ventricular wet weight (LV) (A) and infarct tissue (area of necrosis, AN) as a per cent of the area-at-risk (B) for both normally fed and cholesterol-fed rabbits treated with Ac-YVAD.cmk (YVAD) or with vehicle are presented. Data are expressed as mean ± s.e.m. *$P < 0.01$, **$P < 0.001$.

DNA fragmentation (DNA ladder) in hearts subjected to ischaemia and reperfusion

Myocardial DNA fragmentation in the non-ischaemic and ischaemic non-necrotic areas in all four groups is shown in Figure 4. Ischaemia and reperfusion did not cause any visible DNA ladders in the non-ischaemic area in all groups. Typical nucleosomal DNA ladders indicative of apoptosis were clearly demonstrated in myocardial specimens sampled from the ischaemic non-necrotic area of both vehicle-treated normally fed and cholesterol-fed rabbits. The intensity of DNA ladders was more pronounced in cholesterol-fed rabbits. Treatment with Ac-YVAD.cmk resulted in the disappearance of DNA laddering in the ischaemic non-necrotic area in both normally fed and cholesterol-fed groups.

Caspase-1 and caspase-3 activity after ischemia and reperfusion

To evaluate whether caspase-1 activity was inhibited after a single postischaemic administration of Ac-YVAD.cmk, we assessed enzyme activity in myocardial homogenates 4 h after reperfusion. Caspase-1 activity in the non-ischaemic myocardium was similar between normally fed and cholesterol-fed rabbits. For vehicle-treated normally fed rabbits, there was a 2 fold increase in caspase-1 activity in the ischaemic myocardium compared to the non-ischaemic myocardium (Figure 5A). However, for vehicle-treated cholesterol-fed rabbits, there was a much greater 3.3 fold increase in caspase-1 activity in the ischaemic myocardium compared to the non-ischaemic myocardium. In both the Ac-YVAD.cmk-treated normally fed and cholesterol-fed groups, caspase-1 activity in the ischaemic myocardium was significantly suppressed compared to the vehicle-treated groups. Caspase-1 activity in the ischaemic myocardium was similar between both Ac-YVAD.cmk treated normally fed and cholesterol-fed groups.

We also analysed the activity of caspase-3 after Ac-YVAD.cmk administration. Like caspase-1, compared to the non-ischaemic myocardium, caspase-3 activity in the ischaemic myocardium was markedly increased 4 h after reperfusion in both vehicle-treated normally fed and cholesterol-fed groups. Treatment with Ac-YVAD.cmk sig-

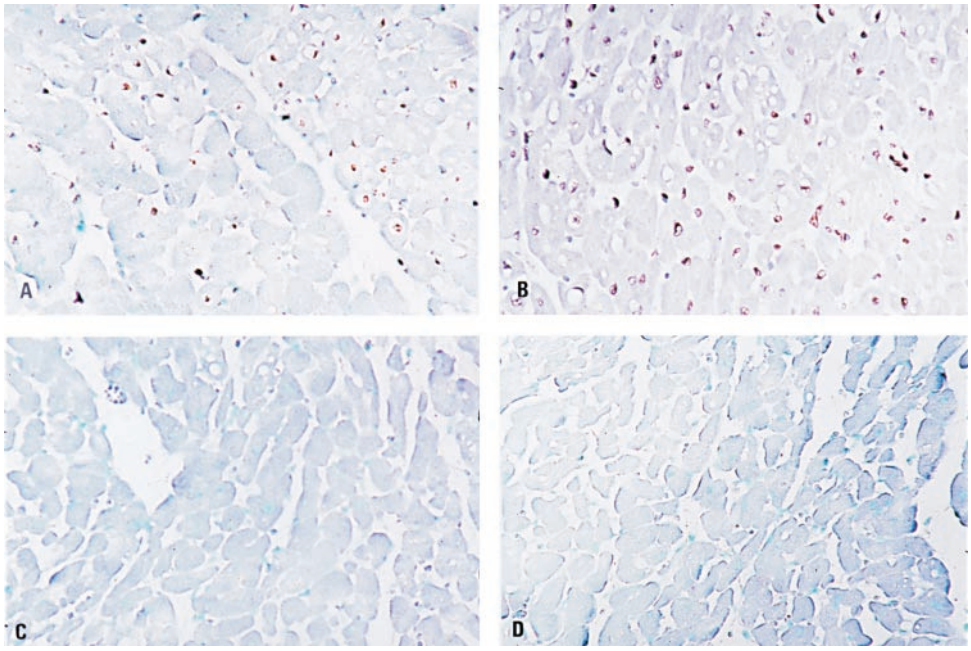

Figure 2 Paraffin sections stained with TUNEL and methyl green in the ischaemic non-necrotic area of vehicle-treated normally fed rabbits (A), vehicle-treated cholesterol-fed rabbits (B), Ac-YVAD.cmk-treated normally fed rabbits (C) and Ac-YVAD.cmk-treated cholesterol-fed rabbits (D) are presented (200× original magnification). The apoptotic cardiomyocyte is detected by the brown nuclear staining in contrast to the methyl green stained normal cardiomyocyte nucleus. Figures are representative of at least six separate experiments.

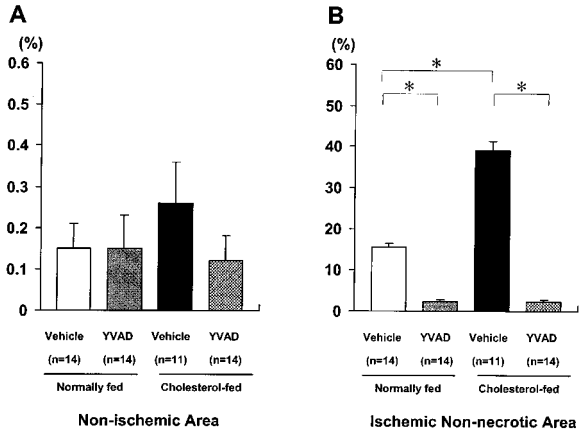

Figure 3 Percentage of cardiomyocyte nuclei stained positive for TUNEL in the non-ischaemic (A) and ischaemic non-necrotic (B) areas from both normally fed and cholesterol-fed rabbits treated with Ac-YVAD.cmk or with vehicle. Data are expressed as mean ± s.e.m. $^*P<0.001$.

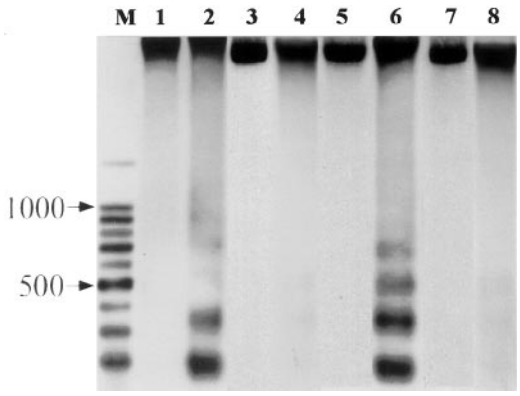

Figure 4 DNA laddering. Laddering represents multiples of 180-bp internucleosomal DNA fragments and corroborates histological evidence of apoptosis. DNA was extracted from the myocardium of vehicle-treated normally fed rabbits (lanes 1 and 2), Ac-YVAD.cmk-treated normally fed rabbits (lanes 3 and 4), vehicle-treated cholesterol-fed rabbits (lanes 5 and 6), and Ac-YVAD.cmk-treated cholesterol-fed rabbits (lanes 7 and 8). Lanes 1, 3, 5, and 7 are from the non-ischaemic area, whereas lanes 2, 4, 6, and 8 are from the ischaemic non-necrotic area. Lane M is 100-bp ladder marker. The figure is representative of at least six separate experiments.

nificantly inhibited caspase-3 activity in the ischaemic myocardium in both normally fed and cholesterol-fed groups compared to the vehicle-treated groups (Figure 5B). Separate experiments on myocardial homogenates indicate that 500 nM Ac-YVAD.cmk inhibited caspase-1 activity by 93%, but had almost no effect on caspase-3 activity.

Interleukin-1β level after ischaemia and reperfusion

Caspase-1 activation leads to processing and release of mature IL-1β. To assess whether Ac-YVAD.cmk inhibition of caspase-1 activity induced also a reduction of IL-1β production, we measured IL-1β levels in the myocardium. For both normally fed and cholesterol-fed rabbits, myocardial IL-1β levels were elevated 4 h after reperfusion and were significantly reduced by Ac-YVAD.cmk treatment (Figure 6). Although myocardial IL-1β levels were significantly higher in vehicle-treated cholesterol-fed rabbits compared to normally fed ones, no differences in myocardial IL-1β levels were

observed between Ac-YVAD.cmk-treated normally fed and cholesterol-fed groups.

Discussion

In this study, we demonstrate that, first, long-term (8 weeks) hypercholesterolaemia significantly exacerbates cardiac reperfusion injury, not only by increasing the infarct size, but also by increasing the extent of cardiomyocyte apoptosis. Likewise, hypercholesterolaemia is associated with markedly increased production of IL-1β and activation of both caspase-1 and caspase-3 in ischaemic-reperfused myocardium. Second, we document that injection of a selective caspase-1 inhibitor (Ac-YVAD.cmk) after myocardial ischaemia reduced myocardial ischaemia-reperfusion injury by attenuating both necrotic as well as apoptotic cell death pathways through inhibition of both IL-1β production and activation of caspase-1 and caspase-3. Furthermore, for the first time it is shown that pharmacological inhibition of caspase-1 cascade markedly reduced the adverse effect conferred by hypercholesterolaemia on myocardial ischaemia-reperfusion injury, i.e., there is no significant difference in either infarct size or the magnitude of cardiomyocyte apoptosis in ischaemic-reperfused myocardium between normally fed and cholesterol-fed rabbits treated with Ac-YVAD.cmk. Our results confirm reports by other groups indicating that hypercholesterolaemia is associated with greater myocardial reperfusion injury (Golino *et al*., 1987; Tilton *et al*., 1987; Osborne *et al*., 1989; Hoshida *et al*., 1999; Wang *et al*., 2002), in which apoptosis plays an important role, and that caspases are activated during this process. The reported data extend previous findings by implicating caspase-1 activation is crucial in mediating post-reperfusion myocardial damage, and by demonstrating marked attenuation of the detrimental effects of hypercholesterolaemia on myocardial reperfusion injury by inhibiting the caspase-1 cascade.

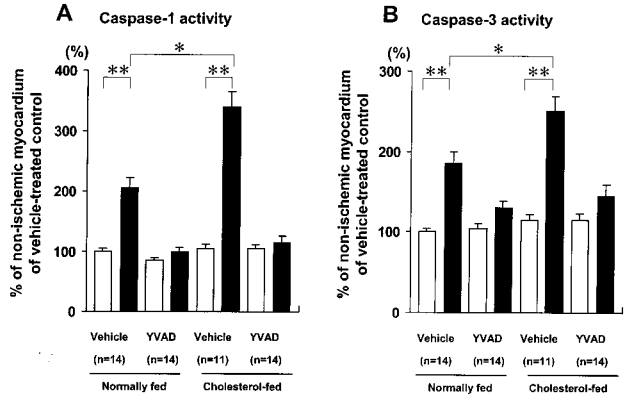

Figure 5 Effects of Ac-YVAD.cmk on caspase-1 and caspase-3 activity in the ischaemic (black) and non-ischaemic (white) myocardium. (A) Caspase-1 activity was determined by measuring cleavage of the fluorogenic substrate Ac-YVAD.aminomethylcoumarin in myocardial homogenates 4 h after reperfusion in rabbits injected intravenously with Ac-YVAD.cmk or vehicle 20 min after coronary occlusion. (B) Caspase-3 activity was measured by the ability of tissue homogenates to cleave DEVD.aminomethylcoumarin. The values for each sample are presented in percentage of control (non-ischaemic myocardium of vehicle-treated normally fed rabbits). Data are expressed as mean$\pm$s.e.m. $^*P<0.05$, $^{**}P<0.001$.

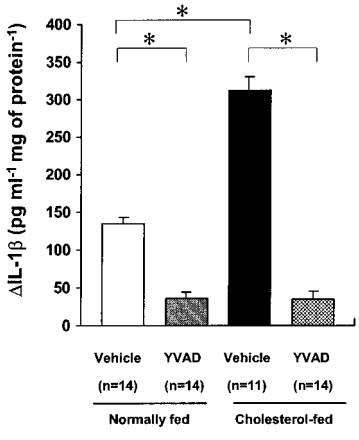

Figure 6 Effect of Ac-YVAD.cmk treatment on IL-1β level. Myocardial IL-1β levels in normally fed and cholesterol-fed rabbits treated with Ac-YVAD.cmk or with vehicle and killed after 30 min of ischaemia and 4 h of reperfusion measured by ELISA assay and expressed as difference between ischaemic and non-ischaemic myocardium. Data shown are expressed as mean$\pm$s.e.m. $^*P<0.001$.

It is noteworthy that rabbits in the cholesterol-fed group had a higher mortality rate following myocardial ischaemia and reperfusion compared with normally fed rabbits. Because rabbits dying in the procedure might have had larger infarct size and a greater extent of cardiomyocyte apoptosis, our observation may underestimate the impact of hypercholesterolaemia on reperfusion-related cardiac injuries. This may partly explain why animals fed a cholesterol-enriched diet for 8 weeks or more did not have markedly increased infarct size following ischaemia and reperfusion than normocholesterolaemic animals in two recent studies (Girod *et al*., 1999; Kremastinos *et al*., 2000). Moreover, we and other investigators have demonstrated that, in the setting of myocardial ischaemia-reperfusion, hypercholesterolaemia is associated with increased inflammatory cell infiltration and cytokine production, which substantially contribute to the development of myocardial reperfusion injury (Ohara *et al*., 1993; Hoshida *et al*., 1999; Wang *et al*., 2002). Therefore, it justifies why isolated perfused heart of the cholesterol-fed animals, in the absence of invading activated inflammatory cells, was not more susceptible to ischaemia-reperfusion injury in some studies (Ferdinandy *et al*., 1997; Le Grand *et al*., 1995).

The extent of cardiomyocyte apoptosis following ischaemia and reperfusion in normocholesterolaemic rabbits reported here agrees with most previous studies, in which 10–15% of cardiomyocytes rendered ischaemic and reperfused were recognized as apoptotic by using the *in situ* TUNEL assay (Gottlieb *et al*., 1994; Olivetti *et al*., 1996; Yaoita *et al*., 2000). We further demonstrate that hypercholesterolaemia is associated with a more than 2 fold increase in the number of apoptotic cardiomyocytes following experimental ischaemia and reperfusion in the ischaemic non-necrotic area ('border zone'). However, it should be pointed out that we might overestimate the magnitude of apoptosis defined by TUNEL staining due to its positive staining of cells in DNA and RNA repair, some necrotic cells, as well as apoptotic cells (Ohno *et al*., 1998; Yaoita *et al*., 2000). Recent studies have demonstrated that in areas where necrotic cells are not

present to any significant amount, good correlation is found between the TUNEL test and other more sophisticated methods (Oskarsson *et al*., 2000). We herein assessed the magnitude of cardiomyocyte apoptosis only in ischaemic areas without evidence of necrosis, confirmed by the absence of background smear on agarose gel electrophoresis. Further studies using the more specific Taq polymerase-based *in situ* ligation assay may resolve this problem (Didenko & Hornsby, 1996).

Caspase activation is a critical process leading to apoptotic cell death. Caspases can be divided on the basis of the substrate specificities and also into functional subfamilies (Denner, 1999). Group I enzymes including caspases-1, -4, -5, and -13 mediate cytokine maturation and inflammation. The apoptotic caspases (groups II and III) are involved in a hierarchically ordering proteolytic cascade. Group III initiators (caspases-8, -6, -9, and -10) act upstream of group II executioners (caspases-3, -7, and -2) that are responsible for the cleavage of crucial substrates in the final degradation phase of the apoptotic process. It is noteworthy that caspase-1, instead of participating in proteolytic processing of several proinflammatory cytokines, has been demonstrated to be responsible for the activation of group II executioner caspases as well (Denner, 1999; Stephanou *et al*., 2000).

Given the dual pro-apoptotic and pro-necrotic properties of caspase-1, we speculated that inhibition of caspase-1 should be of a great value in alleviating myocardial reperfusion injury. Evidence from studies performed with caspase-1 knock-out mice and mice expressing a dominant-negative mutant caspase-1 gene indicates that both of these genetically modified mouse strains are more resistant to cerebral ischaemia-reperfusion insult than wild-type littermates (Hara *et al*., 1997; Schielke *et al*., 1998). Nevertheless, so far there are no reports on the analysis of myocardium in these animals. Ac-YVAD.cmk is an irreversible caspase group I (caspase-1-like) inhibitor mainly active on caspase-1 (K_i 0.8 nM) compared to caspase-4 and caspase-5 (K_i 362 and 163 nM, respectively) (Rabuffetti *et al*., 2000). A previous report has shown that application of Ac-YVAD.cmk prior to ischaemia leads to limitation of infarct size and cardiomyocyte apoptosis in a rabbit myocardial ischaemia-reperfusion model (Holly *et al*., 1999). However, the other study showed that administration of another caspase-1-like inhibitor, Ac-YVAD.aldehyde, before ischaemia did not reduce infarct size in ischaemia-reperfused rat hearts, despite its being effective in reducing the extent of apoptosis (Okamura *et al*., 2000). Reasons for the discrepancy concerning the infarct size reduction between studies remain elusive, and may be related to differences of inhibitors and animal species. Furthermore, studies regarding the roles of various caspase inhibitors in myocardial ischaemia-reperfusion injury have only been undertaken in animal models in which ischaemia is imposed in the absence of other disease processes, despite that ischaemic heart disease in humans is often associated with other systemic diseases such as dyslipidemia, hypertension, and diabetes (Ferdinandy *et al*., 1998).

In the only previous study which demonstrated the cardioprotective effects of Ac-YVAD.cmk by using the same rabbit myocardial ischaemia-reperfusion model as ours, rabbits were given an intravenous bolus of Ac-YVAD.cmk (1.5 mg kg^{-1}) 10 min prior to occlusion, followed by a continuous intravenous infusion (0.5 mg kg^{-1} h^{-1}) until the end of reperfusion, and a second intravenous bolus (1.5 mg kg^{-1}) just prior to reperfusion (Holly *et al*., 1999). Because a plethora of evidence indicates that the apoptotic process is initiated shortly after ischaemia and greatly amplified during reperfusion (Gottlieb *et al*., 1994; Fliss & Gattinger, 1996), we speculated that the cardioprotective effects of Ac-YVAD.cmk observed in that study might be largely achieved by the second intravenous bolus dose given just before reperfusion. In the present study, our experimental setting consisted of a single bolus injection of Ac-YVAD.cmk (1.6 mg kg^{-1}) 10 min prior to reperfusion to simulate the clinical scenario of managing patients with acute myocardial infarction. The dose administered was similar to the second bolus dose given in that previous study. It was demonstrated that under these circumstances Ac-YVAD.cmk still reduced the extent of both cardiomyocyte necrosis and apoptosis in normally fed and diet-induced hypercholesterolaemic rabbits following myocardial ischaemia and reperfusion. Unlike the case of ischaemic preconditioning (Ueda *et al*., 1999), hypercholesterolaemia did not abolish the protective effects of Ac-YVAD.cmk, thus further assuring its potential benefit in hypercholesterolaemic individuals.

In keeping with the finding that hypercholesterolaemic rabbits had a greater magnitude of cardiomyocyte apoptosis than normally fed rabbits following myocardial ischaemia and reperfusion, we have demonstrated that both caspases-1 and -3 were more prominently activated in the reperfused myocardium in hypercholesterolaemic rabbits. Treatment with Ac-YVAD.cmk clearly reduced caspase-1 activity in myocardial homogenates in both normally fed and hypercholesterolaemic rabbits, and the difference of caspase-1 activity between normally fed and hypercholesterolaemic rabbits disappeared with Ac-YVAD.cmk treatment, indicating a greater extent of inhibition of caspase-1 activation in hypercholesterolaemic rabbits. Likewise, Ac-YVAD.cmk markedly reduced the level of IL-1β produced in reperfused myocardium in normally fed and, more dramatically, in hypercholesterolaemic rabbits. These findings suggest that the current dosing of Ac-YVAD.cmk is adequate not only for rabbits without comorbid conditions, but also for those with diet-induced hypercholesterolaemia.

It is noteworthy that caspase-3 was also significantly inhibited by Ac-YVAD.cmk treatment in both normally fed and hypercholesterolaemic rabbits, which is consistent with the observation that caspase-1 is able to directly process procaspase-3 to its active form (Denner, 1999). Although the possibility that Ac-YVAD.cmk *in vivo* may directly inhibit caspase-3 cannot be ruled out, *in vitro* results indicate that the K_i of Ac-YVAD.cmk for caspase-1 (0.8 nM) and caspase-3 ($>10{,}000$ nM) are extremely different (Rabuffetti *et al*., 2000). Furthermore, we observed that relatively high concentration of Ac-YVAD.cmk used for an *in vitro* assay on reperfused myocardial homogenate did not affect caspase-3 activity. In spite of the fact that Ac-YVAD.cmk we used is reported to exert specific and selective anti-caspase effects at the concentration used, its specificity should be accepted with some caution (Mocanu *et al*., 2000). Moreover, we cannot exclude the possibility that Ac-YVAD.cmk might inhibit other proteases, such as calpains, which have been previously implicated in ischaemia-reperfusion injury (Iwamoto *et al*., 1999).

It remains uncertain whether cardiomyocytes exposed to Ac-YVAD.cmk after the initiation of apoptotic signal transduction function normally. These cardiomyocytes may continue to survive or may switch to necrosis because of injury already sustained (LaRue *et al*., 2000). In this study, myocardial infarct size was assessed only 4 h after reperfusion. Despite one recent study showing that neuroprotection conferred by Ac-YVAD.cmk in cerebral ischaemia persisted up to 6 days (Rabuffetti *et al*., 2000), future studies evaluating the viability of cardiomyocytes that escape apoptosis through assessment of infarct extension in the late phase of reperfusion are still warranted.

In conclusion, our data indicate that pharmacological inhibition of caspase-1 as an adjunct to reperfusion results in a significant cardioprotection from ischaemia-reperfusion insult. Furthermore, we present the first evidence that pharmacological inhibition of caspase-1 markedly reduced the detrimental effect conferred by hypercholesterolaemia on myocardial ischaemia-reperfusion injury. This cardioprotective effect is achieved not only by blocking the apoptotic pathway but also by inhibiting IL-1β-mediated inflammation. These observations indicate that inhibition of caspase-1, and more generally caspase-1-like activity, could be a promising therapeutic approach to attenuate myocardial damage caused by ischaemia and reperfusion, particularly in those with concomitant hypercholesterolaemia.

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