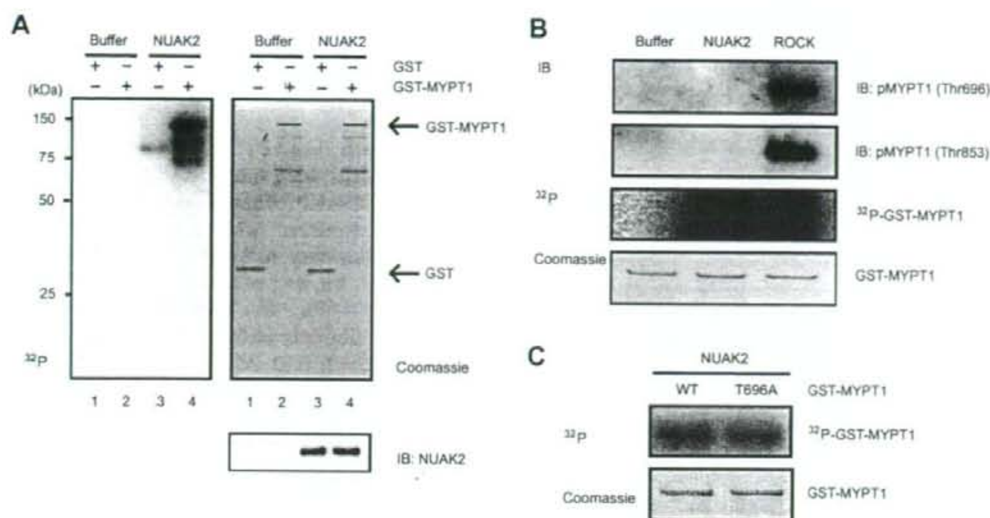

Fig. 4. NUAK2 phosphorylates MYPT1 at another site(s), other than known ROCK phosphorylation sites. (A) GST-alone (lanes 1 and 3) or GST-MYPT1 (lanes 2 and 4) were subjected to an in vitro kinase assay with control (lanes 1 and 2) or GST-NUAK2 (lanes 3 and 4), followed by autoradiography (left panel); Coomassie stain of the same gel (right upper panel), or by immunoblotting with anti-NUAK2 antibody (right lower panel). (B) GST-MYPT1 phosphorylated by recombinant NUAK2 or ROCK was resolved by SDS–PAGE followed by immunoblotting with anti-phosphospecific antibodies against MYPT1 (Thr696 or Thr853), or by autoradiography, or by Coomassie stain, as indicated. These results are representative of three independent experiments. (C) WT or the T696A mutant form of GST-MYPT1 phosphorylated by GST-NUAK2 was resolved by SDS–PAGE followed by autoradiography (upper panel); Coomassie stain of the same gel (lower panel).

known to the major kinase responsible for phosphorylation of MYPT1 [8]. To investigate how the MYPT1 phosphorylation by NUAK2 was regulated, we compared MYPT1 phosphorylation by NUAK2 and that by ROCK. ROCK is known to phosphorylate Thr696 and Thr853 in MYPT1. ROCK phosphorylated MYPT1 at the Thr696 and Thr853, confirmed by immunoblotting with anti-phosphospecific antibodies. Surprisingly, however, NUAK2 did not enhance the phosphorylation of either Thr696 or Thr853 (Fig. 4B). The non-phosphorylatable T696A mutant MYPT1 in which Thr696 was replaced with Ala was phosphorylated by NUAK2 to the same extent as WT-MYPT1 (Fig. 4C). Similarly, the T853A mutant MYPT1 also was phosphorylated by NUAK2 to the same extent as WT-MYPT1 (data not shown). These data suggests that NUAK2 phosphorylates MYPT1 at another site(s), other than known ROCK phosphorylation sites.

Discussion

TNFα is a pleiotropic cytokine that mediates diverse biological responses. To investigate the signal transduction pathways modulated by TNFα and their effect on endothelial cells, the profiles of mRNA expression extracted from HUVECs were analyzed. Most of TNFα-induced genes were chemokine family molecules and chemokine related molecules. We focused on one kinase, NUAK2 among them because it was strongly induced by TNFα. However, none of its substrate has been reported. Knowledge of kinase-substrate relationships is essential to dissect the signaling and regulatory events in which each kinase participates [9]. Direct binding of kinase to its substrate is often reported. Structural analysis reveals that the several binding sites besides catalytic site exist between kinase and its substrate [10]. The kinetics of reactions needs to be enhanced by binding between substrate and kinase. Those affinities might help efficient purification of substrate for target kinase. We successfully purified MYPT1 as a novel substrate for NUAK2 with simple two-step purification. This method is useful for rapid identification of an unknown substrate for certain kinase and can be applied for other substrate screening of kinases.

MYPT1 is a regulatory subunit of myosin phosphatase (MP) which catalyzes dephosphorylation of MLC. Phosphorylation of myosin light chain (MLC) elicits many cellular functions, including smooth muscle contraction [11,12]. MP activity is known to be regulated by upstream kinases. ROCK is most intensively examined [8]. ROCK directly binds MYPT1 and phosphorylates Thr696 and Thr853 of MYPT1 [13]. Phosphorylated MYPT1 by ROCK reduced MP activity resulting increased phosphorylation of MLC [14]. Several other kinases (MYPT1 kinase, integrin-linked kinase and myotonic dystrophy protein kinase) also can phosphorylate the same inhibitory site (Thr696) on MYPT1 [15–17]. The mechanism to reduce MP activity by MYPT1 phosphorylation is still unknown, however, phosphorylation of Thr853 was suggested to directly reduce MYPT1 binding to myosin [18]. Dissociation of the MP holoenzyme by MYPT1 phosphorylation is another suggested mechanism of reduced MYPT1 activ-

ity [19]. NUAK2 did not phosphorylate MYPT1 at either Thr696 or Thr853, confirmed by immunoblotting with anti-phosphospecific antibodies (Fig. 4B) and mutation analysis of MYPT1 (Fig. 4C). These data suggest that NUAK2 phosphorylates MYPT1 at a different site(s) to elicit different regulatory functions. Further characterization of phosphorylation site(s) and elucidation of MYPT1 regulation by NUAK2 are necessary for future study.

Physiological function has been seldom analyzed about NUAK2, however, Legembre et al. reported that NUAK2 works as a part of antiapoptotic signals and an enhancer of cell mobility [4]. If NUAK2 phosphorylates MYPT1 and modulates MLC phosphorylation, these phenotypes would be explained by functional regulation of MYPT1 and its target molecule, myosin. One intriguing thing about NUAK2 is that this is the only kinase highly induced by TNFα. Most of other induced genes were chemokines and adhesion molecules. Chemokine induces chemotaxis of various normal cells, and also plays an important role for invasiveness of tumor cells partly reflected by cell motility. NUAK2-mediated MYPT1 phosphorylation might enhance these chemokine signaling by modifying myosin motor function. In tumor cell lines, TNFα also strongly induces expression of NUAK2, which is related to its motility and invasiveness [4]. These tumor characteristics might be also regulated by NUAK2-mediated myosin motor regulation. The physiological significance of the phosphorylation of MYPT1 by NUAK2 both in normal cells and in tumor cells is now under investigation.

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Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials

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Summary

Background Patients who have acute myocardial infarction remain at major risk of cardiovascular events. We aimed to assess the effects of either human atrial natriuretic peptide or nicorandil on infarct size and cardiovascular outcome.

Methods We enrolled 1216 patients who had acute myocardial infarction and were undergoing reperfusion treatment in two prospective, single-blind trials at 65 hospitals in Japan. We randomly assigned 277 patients to receive intravenous atrial natriuretic peptide (0·025 μg/kg per min for 3 days) and 292 the same dose of placebo. 276 patients were assigned to receive intravenous nicorandil (0·067 mg/kg as a bolus, followed by 1·67 μg/kg per min as a 24-h continuous infusion), and 269 the same dose of placebo. Median follow-up was 2·7 (IQR 1·5–3·6) years for patients in the atrial natriuretic peptide trial and 2·5 (1·5–3·7) years for those in the nicorandil trial. Primary endpoints were infarct size (estimated from creatine kinase) and left ventricular ejection fraction (gauged by angiography of the left ventricle).

Findings 43 patients withdrew consent after randomisation, and 59 did not have acute myocardial infarction. We did not assess infarct size in 50 patients for whom we had fewer than six samples of blood. We did not have angiographs of left ventricles in 383 patients. Total creatine kinase was 66459·9 IU/mL per h in patients given atrial natriuretic peptide, compared with 77878·9 IU/mL per h in controls, with a ratio of 0·85 between these groups (95% CI 0·75–0·97, p=0·016), which indicated a reduction of 14·7% in infarct size (95% CI 3·0–24·9%). The left ventricular ejection fraction at 6–12 months increased in the atrial natriuretic peptide group (ratio 1·05, 95% CI 1·01–1·10, p=0·024). Total activity of creatine kinase did not differ between patients given nicorandil (70520·5 IU/mL per h) and controls (70852·7 IU/mL per h) (ratio 0·995, 95% CI 0·878–1·138, p=0·94). Intravenous nicorandil did not affect the size of the left ventricular ejection fraction, although oral administration of nicorandil during follow-up increased the left ventricular ejection fraction between the chronic and acute phases. 29 patients in the atrial natriuretic peptide group had severe hypotension, compared with one in the corresponding placebo group.

Interpretation Patients with acute myocardial infarction who were given atrial natriuretic peptide had lower infarct size, fewer reperfusion injuries, and better outcomes than controls. We believe that atrial natriuretic peptide could be a safe and effective adjunctive treatment in patients with acute myocardial infarction who receive percutaneous coronary intervention.

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Introduction

Despite availability of effective medical treatments, chronic heart failure remains a major cause of morbidity and mortality worldwide.[1-3] Ischaemic heart disease, in turn, is one of the main causes of chronic heart failure.[4] The most important treatment objectives are prevention of acute myocardial infarction, and, in individuals who have an acute myocardial infarction, reduction in infarct size and ischaemia or reperfusion injury.[5] Only a few medications have been shown to decrease ischaemia or reperfusion injury.[6-8]

Reperfusion of ischaemic myocardium reduces infarct size and improves left ventricular function, both of which contribute to better clinical outcomes in patients with acute myocardial infarction.[9-11] However, reperfusion can also cause tissue damage.[12] Several drugs have been trialled for the prevention or amelioration of such injuries, but results have not been consistently satisfactory.[13-15] Recently, human atrial natriuretic peptide and nicorandil have both been shown to be effective for reduction of myocardial damage after acute myocardial infarction in basic and clinical studies.[16-18] Atrial natriuretic peptide is a candidate for adjunctive treatment after acute myocardial infarction, because it has been shown to suppress the renin–angiotensin–aldosterone system and endothelin-1, both of which which modulate infarct size and cardiac remodelling.[19] Nicorandil is a combined adenosine triphosphate (ATP)-sensitive potassium channel opener and nitrate preparation that has also shown promise as an adjunctive treatment for acute myocardial infarction. In the clinical setting, however,

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the beneficial effects of atrial natriuretic peptide and nicorandil have only been tested in single-centre studies with small sample sizes.[20–25] The Japan working group studies on acute myocardial infarction for the reduction of necrotic damage by human atrial natriuretic peptide or nicorandil (J-WIND-ANP and J-WIND-KATP, respectively) aimed to assess the value of these drugs as adjuncts to percutaneous coronary intervention for patients with acute myocardial infarction.

Methods

Patients

We have described the protocols for the two trials previously.[26,27] In brief, we recruited patients to two independent, investigator-initiated, investigator-led, multicentre, prospective, randomised, single-blind, controlled trials at 65 hospitals. 27 hospitals participated in the atrial natriuretic peptide trial, and 38 separate hospitals in the nicorandil trial (table 1); the two studies were completely independent. We initially planned to include fewer hospitals, but we increased the number to promote enrolment of sufficient patients.

Eligibility criteria were age between 20 and 79 years; chest pain for more than 30 min; at least 0·1 mV of ST segment elevation in two adjacent ECG leads; admission to hospital within 12 h of the onset of symptoms; and one instance of acute myocardial infarction. Exclusion criteria were a history of myocardial infarction; left main trunk stenosis; severe liver or kidney dysfunction or both; suspected aortic dissection; previous coronary artery bypass grafting; and a history of drug allergy.

All patients gave written informed consent immediately after admission to hospital, and were asked to sign the same consent form again after 2 weeks when they had more time to decide. This system was applied on the recommendation of the institutional review boards. Only one patient, who was in the nicorandil group, withdrew their consent at their second opportunity. We enrolled patients from Oct 24, 2001, to Dec 13, 2005. The study protocol was approved by the institutional review boards and ethics committees of all participating hospitals, and was in accordance with the Declaration of Helsinki.

Procedures

An independent statistician generated our randomisation lists with a computer, by the permuted-block method. Within each centre, the block length was eight. Treatment allocations were concealed in opaque sealed envelopes until patients were enrolled. Physicians were not aware of the random assignments of patients until the follow-up stage; patients and those who analysed the data were unaware of the treatment assignment for the duration of the study. Both trials were designed as single-blind studies.

277 patients who were enrolled in the atrial natriuretic peptide trial were randomly assigned to receive an intravenous infusion of this drug after reperfusion treatment, at 0·025 μg/kg per min for 3 days, and 292 a placebo of 5% glucose solution by the same method. 276 patients in the other trial were randomly assigned to intravenous nicorandil, infused at 1·67 μg/kg per min for 24 h after bolus injection of nicorandil at a dose of 0·067 mg/kg, and 269 were assigned to 0·9% saline solution, by the same method. Previous studies have shown substantial cardiovascular protection with atrial natriuretic peptide and nicorandil at these doses.[20,22] Of the 276 patients assigned to receive nicorandil, 61 were given nicorandil orally, at the discretion of individual investigators, during the follow-up period.

We planned to stop the administration of treatment drugs in case of severe hypotension, which was defined as systolic blood pressure of less than 90 mm Hg, because of the vasodilator effect of these drugs. The study protocol did not restrict or specify any other diagnostic or therapeutic methods in the acute phase (2–8 weeks after acute myocardial infarction) or chronic phase (6–12 months).

We obtained data on baseline characteristics, emergent catheterisation, and medication at discharge after 1 month; data on follow-up catheterisation and medication after 6 months; and data on medication after 24 months. We also followed up all patients for cardiovascular events (ie, cardiac death, readmission to hospital due to heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions) until the end of August, 2006. We took blood samples to measure concentrations of creatine kinase at a central laboratory, before the procedure and at 1, 3, 6, 9, 12, 18, 24, 36, 48, and 72 h after the onset of reperfusion.[14] We analysed total creatine kinase for all patients with at least six blood samples. We obtained right anterior oblique views with angiography of the left ventricle once in the acute phase (2–8 weeks), and once in the chronic phase (6–12 months).

Our primary endpoints were infarct size (which was estimated as the area under the concentration versus time curve for creatine kinase)[14] and ventricular ejection fraction (which was assessed by angiography of the left ventricle at 6–12 months after hospital admission).[15] The prespecified secondary endpoints were survival rate; cardiovascular events (such as cardiac death, readmission to hospital for heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions); incidence of cardiac death or readmission to hospital for

	J-WIND-ANP study	J-WIND-KATP study
1–4 patients	7 hospitals	9 hospitals
5–9 patients	3 hospitals	13 hospitals
10–19 patients	7 hospitals	6 hospitals
More than 20 patients	10 hospitals	10 hospitals

Table 1: Distribution of patients between participating hospitals

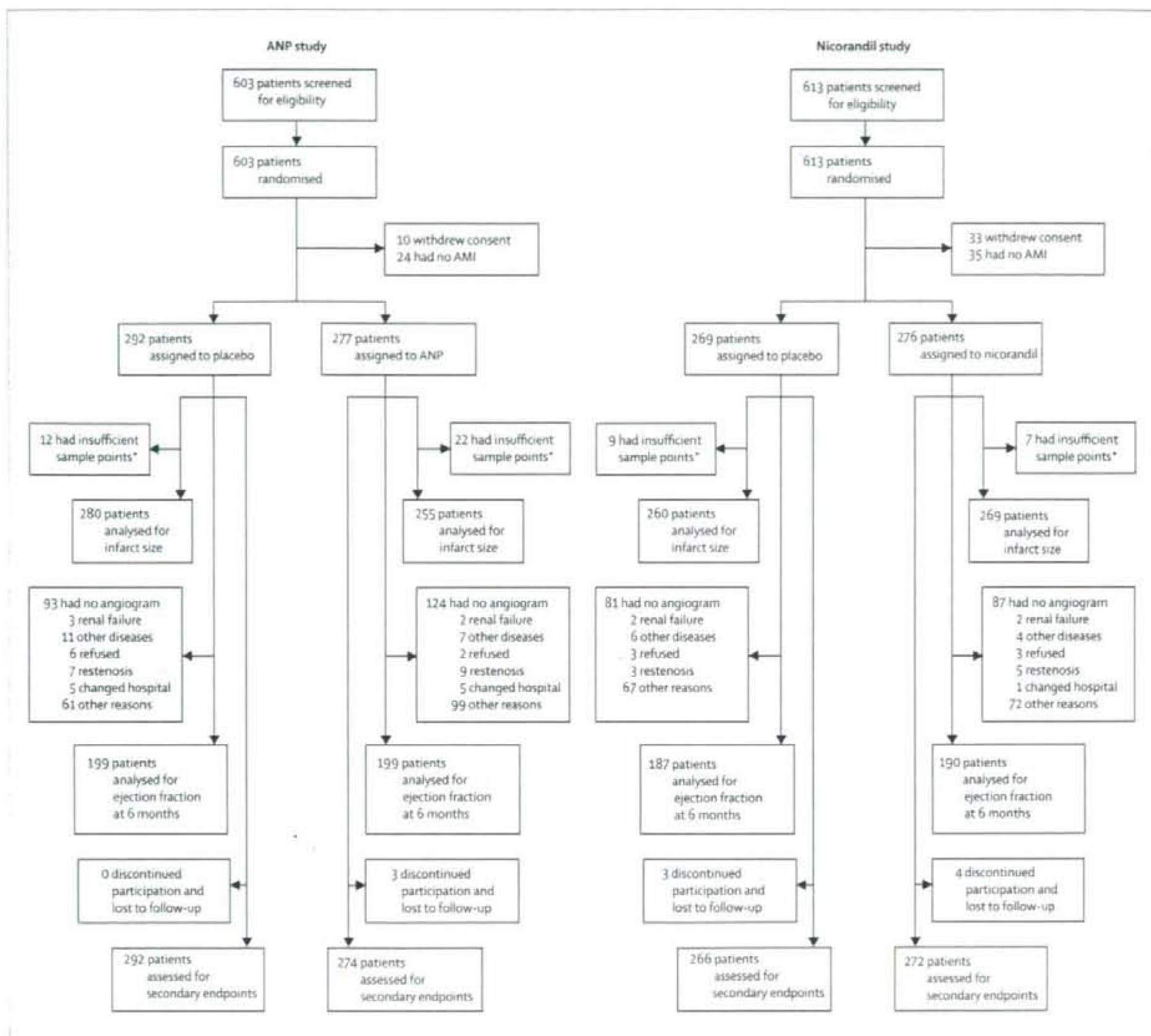

Figure 1: Trial profiles

ANP=atrial natriuretic peptide. AMI=acute myocardial infarction. *Fewer than six blood samples.

heart failure; or reperfusion injury before discharge from coronary care unit (such as malignant ventricular arrhythmia during reperfusion, recurrence of ST segment elevation, or worsening of chest pain). We also assessed infarct size, estimated by peak creatine kinase and troponin T;[28,29] left ventricular ejection fraction at acute phase; and end-diastolic or end-systolic volume index (assessed by angiography of the left ventricle). We looked at the effects of each drug on the primary endpoints in prespecified subgroups (sex, age, body-mass index, pre-angina, elapsed time between acute myocardial infarction and intervention, diabetes mellitus, hyperlipidaemia, smoking, and family history of acute myocardial infarction). We also did post-hoc analyses on the effect of chronic administration of nicorandil on the ejection fraction.

All data were collected by Koteisho-kyokai (Tokyo), an organisation established by the Japanese government in 2001–2003 and by NTT Data (Tokyo) in 2004–2006. Left ventricular ejection fraction and end-diastolic volume were measured by the area–length method, from angiography of the left ventricle. Two independent

interpreters, who were unaware of the treatment assigned to patients, measured left ventricular ejection fractions from the angiographs. We calculated the average value, unless the two investigators disagreed, in which case we referred to a third opinion.

Clinical findings and medications during the follow-up period were reported to a data and safety committee after registration. This committee, which consisted of three physicians and one statistician who did not participate in the trial, monitored all adverse events. Research nurses or doctors visited all participating hospitals to check that patients were registered, drugs were given, and data collected according to the protocol. Committee members did not provide any results to the steering committee, because discontinuation of the study was not recommended.

Statistical analysis

We calculated that a sample size of 300 patients would be needed in each group to detect a 20% reduction in the most important primary endpoint (total creatine kinase) with a statistical power of 80% at significance level of 0·05 (with a two-sided *t* test), accounting for dropout of some patients. We set equal sample sizes in both groups, because we expected to see almost the same reduction in infarct size with either treatment. Since creatine kinase and total creatine kinase are both log-normally distributed,[30] total creatine kinase was log-transformed before analysis. The left ventricular ejection fraction was also log-transformed before the analysis since the distribution was skewed.

Statistical analysis was done according to a prespecified analytical plan. Efficacy analysis was based on intention to treat. The primary efficacy analyses for total creatine kinase and left ventricular ejection fraction were done simply by *t* test. The estimated mean and differences on the log scale were transformed back to the original scale and were expressed as geometric means and ratios of geometric mean. If the calculated 95% CI for the ratio of the geometric mean did not cross the point of no effect (ie, 1) the difference between groups was regarded as significant. Furthermore, analysis of covariance for the two endpoints was used to estimate adjusted mean comparison, with effect of covariates and the interactions. We imputed missing data for patients by the predicted mean imputation method, with nonlinear regression. We applied multiple imputation techniques (with group means, Markov Chain Monte Carlo, Bayesian bootstrap, and last-observation-carried-forward methods) to assess the robustness and sensitivity of our conclusions.

Proportions were examined by Fisher's exact test. We examined time-to-event by the Kaplan-Meier method to estimate the survival for each group and then the differences in survival between groups by the log-rank test. The Cox proportional hazards model was used to assess baseline risk factors and an adjusted hazard ratio. The proportional hazards assumption was investigated graphically, with a test based on Schoenfeld residuals.[31,32]

All tests were two-sided, and a p value of less than 0·05 was regarded as significant. All analyses were done with SAS software (version 8.2). The trials are registered with Clinicaltrials.gov, numbers NCT00212056 and NCT00212030.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data at the end of the study, and had final responsibility for the decision to submit for publication.

Results

Figure 1 shows the trial profile. Table 2 shows baseline characteristics. Median follow-up was 2·7 (IQR 1·5–3·6) years in the atrial natriuretic peptide trial and 2·5 (1·5–3·7) years in the nicorandil trial. Table 3 shows

	Atrial natriuretic peptide study			Nicorandil study		
	ANP (n=277)	Control (n=292)	p	Nicorandil (n=276)	Control (n=269)	p
Age (years)	63·0 (10·4)	61·8 (10·7)	0·1652	61·1 (11·4)	63·7 (10·2)	0·0035
Sex (male)	211 (76·2%)	243 (83·2%)	0·0374	246 (89·1%)	220 (81·8%)	0·0153
Body-mass index	24·3 (3·5)	24·0 (2·9)	0·3733	24·2 (3·0)	23·4 (2·8)	0·0007
Killip classification (I, II, III, IV)	88·6%, 9·5%, 1·1%, 0·8%	90·3%, 7·5%, 1·4%, 0·7%	0·5274	91·1%, 8·2%, 0·4%, 0·4%	92·0%, 4·2%, 2·7%, 1·1%	0·7843
Pre-angina	105 (44·5%)	118 (46·1%)	0·7862	111 (44·6%)	111 (43·9%)	0·9284
Risk factors						
Hypertension	137 (56·1%)	162 (62·1%)	0·2046	127 (48·5%)	137 (53·9%)	0·2190
Diabetes mellitus	81 (33·8%)	86 (33·9%)	1·0000	104 (39·5%)	82 (32·9%)	0·1413
Hyperlipidaemia	127 (54·3%)	131 (50·6%)	0·4181	121 (46·7%)	114 (46·2%)	0·9291
Smoking	158 (63·7%)	175 (67·3%)	0·4022	178 (68·7%)	170 (66·1%)	0·5732

Data are number (%) or mean (SD), unless otherwise specified. ANP=atrial natriuretic peptide.

Table 2: **Baseline characteristics on admission**

	Atrial natriuretic peptide study		Nicorandil study	
	ANP (n=277)	Control (n=292)	Nicorandil (n=276)	Control (n=269)
Elapsed time (h)*	4·00 (3·00–6·00)	4·00 (2·50–6·00)	3·50 (2·50–5·00)	3·50 (2·50–5·00)
Infusion time (h)	1·00 (0·50–1·00)	1·00 (0·50–1·00)	0·70 (0·50–1·00)	0·75 (0·50–1·00)
IRA (LAD, LCx, RCA)	55·3%, 6·4%, 38·3%	52·3, 10·6, 37·1%	53·9, 7·4, 38·7%	44·5, 9·9, 45·6%
Stents	176 (63·5%)	193 (66·1%)	187 (67·8%)	183 (68·0%)
Rescue	64 (23·1%)	92 (31·5%)	94 (34·1%)	92 (34·2%)
Intra-aortic balloon pump	17 (6·1%)	14 (4·8%)	14 (5·1%)	15 (5·6%)
Final stenosis (<75%)	246 (93·5%)	266 (94·7%)	257 (96·6%)	255 (97·0%)
Final thrombolysis in myocardial infarction (0, 1, 2, 3)	3·9%, 1·9%, 5·0%, 89·1%	5·2%, 0·7%, 4·1%, 90·0%	3·7%, 0·7%, 5·2%, 90·3%	3·4%, 1·1%, 6·9%, 88·5%
Medications at 1 month				
ACE inhibitor	155 (57·8%)	173 (60·7%)	164 (61·0%)	163 (62·0%)
ARB	77 (28·7%)	99 (34·7%)	72 (26·8%)	69 (26·2%)
Spinorolactone	28 (10·4%)	33 (11·6%)	17 (6·3%)	22 (8·4%)
β blocker	112 (41·8%)	128 (44·9%)	110 (40·9%)	121 (46·0%)
Aspirin	225 (84·0%)	252 (88·4%)	251 (93·3%)	250 (95·1%)
Nitrates	81 (30·2%)	86 (30·2%)	50 (18·6%)	63 (24·0%)
Statins	129 (48·1%)	156 (54·7%)	126 (46·8%)	115 (43·7%)
Nicorandil	62 (23·1%)	52 (18·2%)	79 (29·4%)	34 (12·9%)
Medications at 6 months				
ACE inhibitor	103 (48·1%)	117 (44·8%)	120 (50·6%)	131 (53·9%)
ARB	69 (32·2%)	110 (42·1%)	68 (28·7%)	75 (30·9%)
Spinorolactone	26 (12·1%)	26 (10·0%)	11 (4·6%)	15 (6·2%)
β blocker	93 (43·5%)	118 (45·2%)	104 (43·9%)	113 (46·5%)
Aspirin	179 (83·6%)	233 (89·3%)	217 (91·6%)	229 (94·2%)
Nitrates	51 (23·8%)	63 (24·1%)	37 (15·6%)	49 (20·2%)
Statins	112 (52·3%)	150 (57·5%)	123 (51·9%)	118 (48·6%)
Nicorandil	46 (21·5%)	39 (14·9%)	55 (23·2%)	23 (9·5%)
Medications at 24 months				
ACE inhibitor	66 (47·5%)	63 (37·5%)	83 (52·5%)	75 (49·3%)
ARB	42 (30·2%)	72 (42·9%)	39 (24·7%)	43 (28·3%)
Spinorolactone	13 (9·4%)	21 (12·5%)	9 (5·7%)	4 (2·6%)
β blocker	57 (41·0%)	61 (36·3%)	77 (48·7%)	71 (46·7%)
Aspirin	113 (81·3%)	133 (79·2%)	143 (90·5%)	137 (90·1%)
Nitrates	29 (20·9%)	45 (26·8%)	23 (14·6%)	25 (16·4%)
Statins	66 (47·5%)	78 (46·4%)	81 (51·3%)	71 (46·7%)
Nicorandil	26 (18·7%)	26 (15·5%)	28 (17·7%)	11 (7·2%)

Data are median (IQR), number (%) or mean (SD), unless otherwise specified. ANP=atrial natriuretic peptide. IRA=infarct-related artery. LAD=left anterior descending coronary artery. LCx=left circumflex artery. RCA=right coronary artery. ARB=antiotensin receptor blocker. ACE=angiotensin-converting enzyme. *Period between acute myocardial infarction and start of intervention.

Table 3: **Treatments and prescribed drugs**

treatments and drugs throughout the study. Drugs used in the chronic stage did not differ between groups in either study, except that some patients in the nicorandil trial were given oral nicorandil during follow-up.

Table 4 and figure 2 show infarct size and left ventricular function at 2–8 weeks and 6–12 months in both studies. The ratio of total creatine kinase between the atrial natriuretic peptide and placebo groups was 0·85 (95% CI 0·75–0·97, p=0·0155); which indicates that atrial natriuretic peptide was associated with a reduction of 14·7% in infarct size. Subanalyses identified no factors that enhanced or reduced the influence of atrial natriuretic peptide on infarct size (figure 2). Nicorandil did not reduce infarct size compared with placebo, and no factors affected this finding. Treatment with atrial natriuretic peptide tended to increase the left ventricular ejection fraction (ratio 1·043, 95% CI 1·000–1·089, p=0·0525) at 2–8 weeks after the onset of acute myocardial infarction, and at 6–12 months (ratio 1·051, 95% CI 1·006–1·099, p=0·0236). By contrast, table 4 and figure 2 show that left ventricular ejection fraction did not differ in patients given nicorandil and controls at either 2–8 weeks or 6–12 months.

	J-WIND-ANP study			J-WIND-KATP study		
	Atrial natriuretic peptide	Control	p	Nicorandil	Control	p
Infarct size						
n	255	280		269	260	
Creatine kinase (area under curve) (IU/L h)	66 459·9 (60 258·2–73 300·0)	77 878·9 (71 590·2–84 720·1)	0·016	70 520·5 (64 309·8–77 331·0)	70 852·7 (65 066·7–77 153·2)	0·941
Peak creatine kinase (IU/L)	2487·5 (2217·6–2790·3)	2784·2 (2526·7–3067·9)	0·141	2557·1 (2306·1–2835·4)	2428·7 (2199·8–2681·5)	0·479
Troponin-T concentration (12–18 h) (ng/mL)	5·36 (4·76–6·03)	6·13 (5·55–6·79)	0·084	6·18 (5·51–6·93)	5·60 (4·97–6·32)	0·244
Troponin T (96 h) (ng/mL)	2·57 (2·25–2·94)	2·94 (2·64–3·27)	0·125	2·63 (2·36–2·94)	2·89 (2·61–3·19)	0·225
Left ventricle (2–8 weeks)						
n	187	207		168	170	
Median elapsed time (days)*	18·5 (IQR 15·0–27·0)	19·0 (IQR 16·0–25·0)		17·0 (IQR 14·0–23·0)	17·0 (IQR 14·0–24·0)	
Ejection fraction	43·0% (41·8–44·3)	41·3% (40·0–42·6)	0·053	42·0% (40·7–43·3)	41·6% (40·4–42·9)	0·680
End diastolic volume index (mL/m²)	98·8 (94·4–103·4)	102·3 (98·1–106·6)	0·272	111·2 (106·4–116·3)	105·9 (100·9–111·3)	0·147
End systolic volume index (mL/m²)	54·2 (51·2–57·4)	58·3 (55·5–61·4)	0·058	62·8 (59·2–66·6)	60·4 (57·0–64·1)	0·360
Left ventricle (6–12 months)						
n	155	199		190	187	
Median elapsed time (days)*	196·5 (IQR 180·5–230·5)	200·5 (IQR 183·0–226·0)		195·0 (IQR 180·0–231·0)	195·5 (IQR 183·0–232·0)	
Ejection fraction	44·7% (43·4–46·0)	42·5% (41·2–43·9)	0·024	42·5% (41·2–43·8)	43·2% (42·0–44·4)	0·460
End diastolic volume index (mL/m²)	100·6 (95·2–106·2)	100·9 (96·8–105·1)	0·930	109·8 (105·4–114·4)	105·7 (100·8–110·8)	0·230
End systolic volume index (mL/m²)	54·2 (50·6–58·0)	56·0 (53·1–58·9)	0·452	61·7 (58·4–65·2)	58·5 (55·1–62·1)	0·198

Data are mean (95% CI) or median (IQR). *Time between acute myocardial infarction and start of intervention.

Table 4: **Primary endpoints and other outcomes obtained by angiography of left ventricles**

Figure 3 shows reperfusion injuries, survival rates, and cardiovascular events. Reperfusion injuries were less common in the atrial natriuretic peptide group than in the placebo group (ratio 0·743, 95% CI 0·58–0·952, p=0·019). Although there were no differences between groups in either survival rates or the incidence of cardiovascular events, both cardiac death and readmission to hospital for heart failure were lower in patients given atrial natriuretic peptide than in controls (HR 0·267, 95% CI 0·089–0·799, p=0·0112). By contrast, cardiac death and readmission to hospital for heart failure were not significantly lower in patients given nicorandil than in controls (HR 0·799, 95% CI 0·307–1·973, p=0·5972). When nicorandil was given orally throughout the study after reperfusion treatment, the change of left ventricular ejection fraction increased substantially between the acute and chronic phase. The ejection fraction was 3·66% in the 61 patients who were given nicorandil orally, and 1·47% in the 241 patients who were not (difference 2·20, 95% CI 0·17–4·22, p=0·0338).

In the atrial natriuretic peptide trial, 29 patients given that drug had severe hypotension during the acute phase, compared with one control. In the other trial, three patients in the nicorandil group had severe hypotension, compared with no controls. No other severe adverse events were reported during the course of either study.

Discussion

We showed that adjunctive, acute-phase treatment with atrial natriuretic peptide after reperfusion therapy in patients with acute myocardial infarction reduced infarct size by 14·7%, increased the left ventricular ejection fraction during the chronic phase, and decreased the incidence of cardiac death and readmission to hospital because of heart failure. Intravenous treatment with nicorandil did not affect the primary endpoints, although patients who were given nicorandil orally had better cardiac function outcomes.

Interest in the cardioprotective effects of adenosine has increased, because of its variety of cardioprotective mechanisms. Unfortunately, in trials of adenosine, it only marginally improved infarct size and showed no clinical benefits.[7,33] We hypothesised that treatment with atrial natriuretic peptide and nicorandil in the acute phase might prove more effective than chronic-phase treatment for limitation of infarct size. The first window of ischaemic preconditioning is mediated by opening of the KATP channel,[34] which is the mechanism of action of nicorandil; and the second window is mediated by nitric oxide and activation of G kinase, which is the mechanism of action of atrial natriuretic peptide.

Before this clinical trial, we had tested whether atrial natriuretic peptide could limit infarct size in a canine model in which the left anterior coronary artery was ligated for 90 min, followed by 6 h of reperfusion. Treatment with atrial natriuretic peptide reduced infarct size by about 40% after reperfusion (unpublished data). Our results are consistent with the finding of Hayashi and coworkers[20] that infusion of atrial natriuretic peptide immediately after reperfusion in patients with their first anterior acute myocardial infarction increased left ventricular ejection fraction.

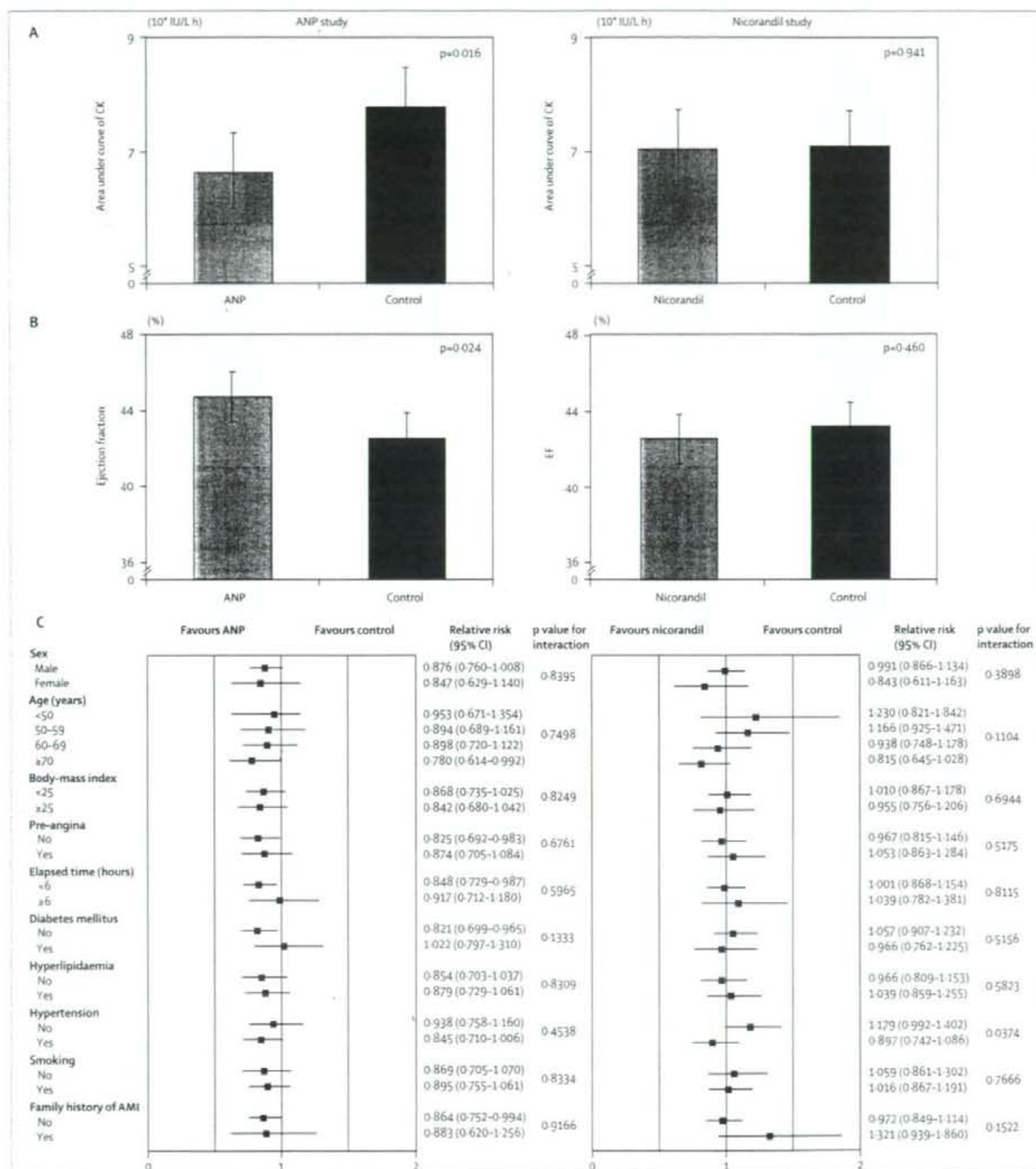

***Figure 2:* Primary endpoints and subgroup analyses**

CK=creatine kinase. AMI=acute myocardial infarction. ANP=atrial natriuretic peptide. Panel A shows area under curve of creatine kinase concentration versus time. Panel B represents left ventricular ejection fraction measured at 6–12 months.

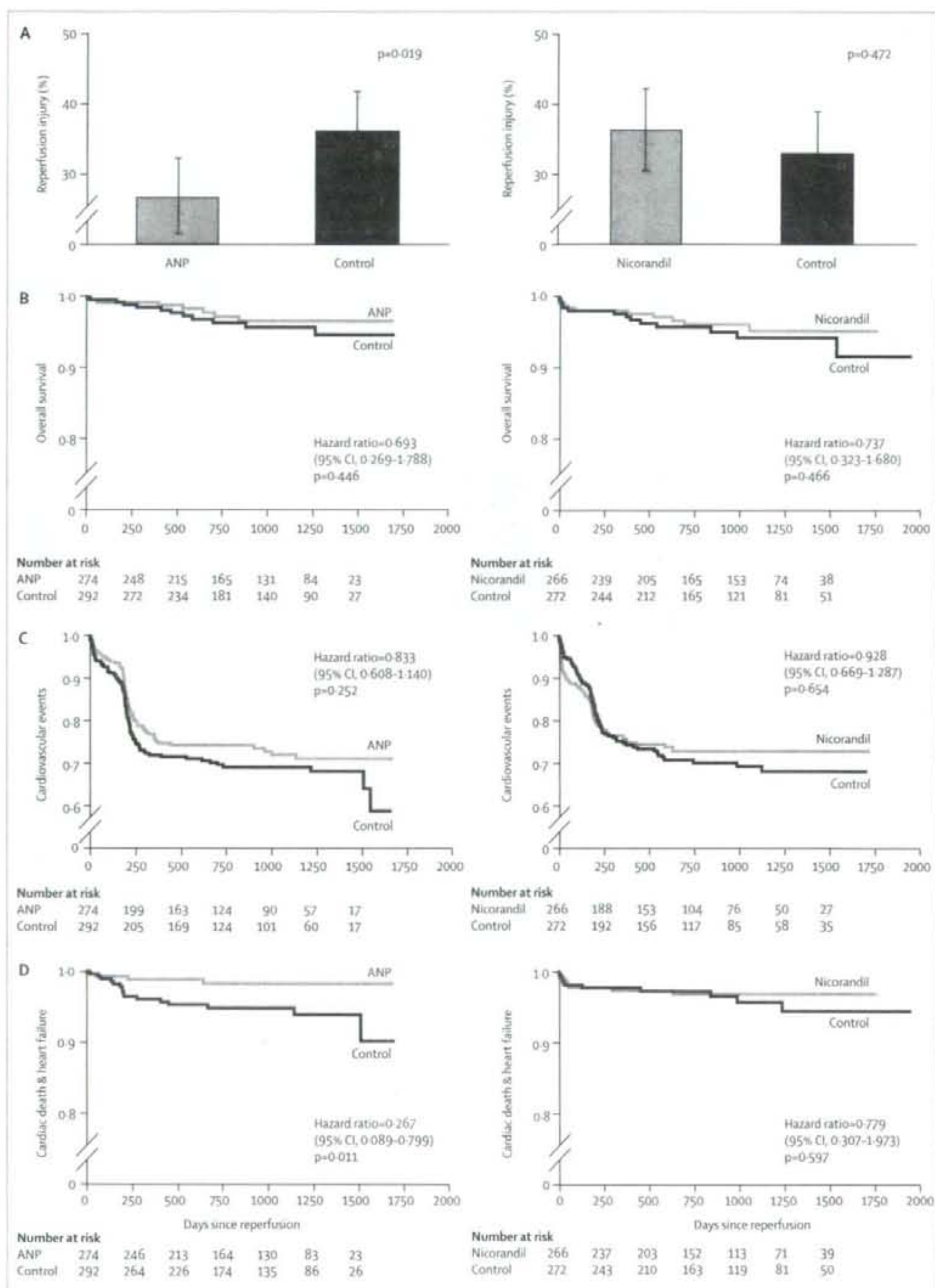

***Figure 3:* Secondary endpoints and other subanalyses**
ANP=atrial natriuretic peptide

The reduction of infarct size and the improvement of left ventricular ejection fraction might decrease mechanical stress on the non-infarcted myocardium, which might decrease hypertrophy and dilatation of the non-infarcted myocardium. Since cardiac hypertrophy and dilatation cause diastolic and systolic heart failure, a reduction of infarct size and an increase of left ventricular ejection fraction could mediate beneficial clinical outcomes. However, we need to do another large-scale clinical trial to target clinical outcomes such as cardiovascular death, because our primary aim here was to test the reduction of infarct size. Moreover, Hayashi and colleagues[20] showed that plasma concentrations of angiotensin II, aldosterone, and endothelin-1 were lower in patients given atrial natriuretic peptide than in controls. Sudden exposure to high concentrations of angiotensin II, aldosterone, and endothelin-1 for several days caused vascular or ventricular remodelling, and attenuation of these harmful effects by infusion of atrial natriuretic peptide could reduce the incidence of cardiac death and readmission to hospital for chronic heart failure.[20]

One reason that nicorandil treatment did not limit infarct size in our study could be the size of the dose. Ishii and colleagues[21] have reported that one intravenous administration of a dose of nicorandil that was three times higher than that which we used decreased the infarct size and reduced the rate of cardiovascular death or readmission to hospital for chronic heart failure in 368 patients with acute myocardial infarction.

Patients in the nicorandil study who were given nicorandil orally in the chronic phase had greater increases in left ventricular ejection fraction, irrespective of whether nicorandil was given intravenously or orally. Since microvascular obstruction ten days after myocardial infarction was associated with left ventricular remodelling and poor prognosis, coronary perfusion might be improved by opening KATP channels in coronary blood vessels during the healing stage. The IONA study[35] showed that nicorandil could reduce the incidence of unstable angina in patients with stable angina.

Our finding that treatment with atrial natriuretic peptide in the acute phase reduced the incidence of readmission to hospital for chronic heart failure could help to reduce the physical, medical, and economic burdens on people around the world. Moreover, since intravenous nicorandil in the acute phase, followed by oral administration in the chronic phase, increased the left ventricular ejection fraction, chronic treatment with nicorandil could improve ventricular function for patients with myocardial infarction in the chronic phase.

Several limitations of our study should be discussed. First, physicians knew the random assignment of patients, and treatment for acute myocardial infarction in the chronic phase was not restricted accordingly; this could have affected the difference in nicorandil treatment at the chronic phase. Second, although we planned to do angiography of the left ventricle when patients were admitted to hospital, some hospitals could not take angiographs, because of the additional medical cost. Therefore, baseline angiographs were absent for some patients. Third, the patterns of missing angiography data on left ventriculography differed between the two studies (which were done at different hospitals) and also between the atrial natriuretic peptide group and corresponding placebo group. We cannot explain this difference, but since we did not intervene in this procedure, we believe that it must be due to chance.

Contributors

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Conflict of interest statement

We declare that we have no conflict of interest.

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Increased Endoplasmic Reticulum Stress in Atherosclerotic Plaques Associated With Acute Coronary Syndrome

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Background—The endoplasmic reticulum (ER) responds to various stresses by upregulation of ER chaperones, but prolonged ER stress eventually causes apoptosis. Although apoptosis is considered to be essential for the progression and rupture of atherosclerotic plaques, the influence of ER stress and apoptosis on rupture of unstable coronary plaques remains unclear.

Methods and Results—Coronary artery segments were obtained at autopsy from 71 patients, and atherectomy specimens were obtained from 40 patients. Smooth muscle cells and macrophages in the fibrous caps of thin-cap atheroma and ruptured plaques, but not in the fibrous caps of thick-cap atheroma and fibrous plaques, showed a marked increase of ER chaperone expression and apoptotic cells. ER chaperones also showed higher expression in atherectomy specimens from patients with unstable angina pectoris than in specimens from those with stable angina. Expression of 7-ketocholesterol was increased in the fibrous caps of thin-cap atheroma compared with thick-cap atheroma. Treatment of cultured coronary artery smooth muscle cells or THP-1 cells with 7-ketocholesterol induced upregulation of ER chaperones and apoptosis, whereas these changes were prevented by antioxidants. We also investigated possible signaling pathways for ER-initiated apoptosis and found that the CHOP (a transcription factor induced by ER stress)-dependent pathway was activated in unstable plaques. In addition, knockdown of CHOP expression by small interfering RNA decreased ER stress-dependent death of cultured coronary artery smooth muscle cells and THP-1 cells.

Conclusions—Increased ER stress occurs in unstable plaques. Our findings suggest that ER stress-induced apoptosis of smooth muscle cells and macrophages may contribute to plaque vulnerability. (***Circulation*. 2007;116:1226-1233.**)

Key Words: apoptosis ■ plaque ■ myocardial infarction ■ endoplasmic reticulum

Most of the acute clinical manifestations of coronary atherosclerosis result from plaque rupture that triggers thrombosis and vessel occlusion, producing the acute coronary syndrome (ACS).[1–3] Previous reports have shown that apoptosis affects all of the types of cells residing within atherosclerotic plaques, including smooth muscle cells (SMCs) and macrophages,[4,5] with oxidized low-density lipoprotein and several inflammatory factors being known to induce apoptosis.[6,7] The number of apoptotic cells depends on the plaque stage and is generally higher in more advanced plaques.[6,8] SMCs synthesize most of the interstitial collagen that stabilizes the fibrous cap of a plaque.[4,7] Therefore, excessive apoptosis of SMCs in the fibrous cap may compromise plaque integrity and render it vulnerable to proteolytic attack by inflammatory cells, leading to plaque rupture.[4,7] Apoptotic macrophages are more frequent at sites of plaque rupture than in areas where the fibrous cap remains intact.[9] A decrease in macrophages would reduce the scavenging of apoptotic SMCs and macrophages, allowing the cells to undergo secondary necrosis, thereby increasing thrombogenicity of the plaque.[10]

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The endoplasmic reticulum (ER) is 1 of the largest cellular organelles and has multiple functions, such as regulating the folding of proteins.[11,12] Various stimuli cause ER stress,

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including ischemia, hypoxia, heat shock, mutation, increased protein synthesis, and reactive oxygen species, all of which can potentially lead to ER dysfunction.[11,12] In response to ER stress, there is marked upregulation of various ER chaperones, such as the 94-kDa glucose-regulated protein (GRP94) or GRP78 that stabilizes protein folding.[11,13,14] When the ER becomes overloaded with misfolded proteins, the unfolded protein response (UPR) occurs to enhance cell survival.[15] However, prolonged ER stress can trigger apoptotic cell death, which is promoted by transcriptional induction of C/EBP homologous protein (CHOP) and/or by the activation of c-JUN NH_2-terminal kinase (JNK)– and/or caspase-12–dependent pathways.[16] In support of this concept, our investigation of the effects of prolonged ER stress on hypertrophic and failing hearts revealed that apoptosis of cardiac myocytes was induced via activation of CHOP, an ER-specific proapoptotic factor.[17] An important role of ER-initiated cell death pathways has also been demonstrated in several diseases, including diabetes mellitus,[16] neurodegenerative conditions,[18] and ischemia.[19]

Oxidation of low-density lipoprotein plays a significant pathogenetic role in atherosclerosis.[6,7,20] In cultured peritoneal macrophages, excessive accumulation of free cholesterol (induced by acetyl low-density lipoprotein with an acyl-CoA:cholesterol acyltransferase [ACAT] inhibitor) initiates ER stress, increases CHOP expression, and leads to apoptosis.[21] Studies of apo$E^{-/-}$ mice also support the relevance of ER stress to macrophage apoptosis and to enlargement of the necrotic core in advanced atherosclerotic plaques.[22,23] However, it is still unclear whether ER stress and UPR activation have a role in plaque rupture. Unfortunately, the absence of a suitable animal model has greatly hindered investigation of the molecular mechanisms of plaque rupture and evaluation of the effects of ER stress in vivo.[24,25]

In the present study, we examined histological sections from atherosclerotic coronary artery lesions obtained at autopsy or after directional coronary atherectomy (DCA) to investigate markers of ER stress/UPR activation and apoptotic cell death. Oxysterols such as 7-ketocholesterol (7-KC) have been reported to be partially responsible for the cytotoxicity of oxidized low-density lipoprotein.[26,27] Exposure of cultured human SMCs to 7-KC induces the UPR and promotes apoptotic cell death,[28] so we investigated 7-KC expression in plaque specimens by immunohistochemistry. We also examined whether 7-KC could activate ER stress using cultured human coronary artery SMCs (CASMCs) and a monocyte cell line (THP-1). Furthermore, we investigated the possible signaling pathways for ER-initiated apoptosis, and we found that the CHOP (a transcription factor induced by ER stress)-dependent pathway was activated in unstable plaques, whereas knockdown of CHOP expression by small interfering RNA (siRNA) decreased ER stress-dependent death of cultured CASMCs and THP-1 cells.

Methods

Coronary Artery Specimens

Two different sets of specimens were obtained under a protocol approved by the Institutional Review Board of the National Cardiovascular Center and Miyazaki University. The first set of specimens was obtained at autopsy, and the second set was obtained by DCA. Classification of the histology of the lesions in autopsy specimens was done morphologically, as described previously (Table 1).[29–31] Demographic data for the study population are presented in Table I of the Data Supplement.

TABLE 1. Human Coronary Specimens (Autopsy; n=71)

Histological Classification of Lesions	No. of Specimens	AHA Classification
No. of specimens obtained at autopsy	152	...
Diffuse intimal thickening (normal)	21	Type I
Fibrous plaques (fibrous)	48	Type Vc
Thick-cap atheroma (thick)	51	Type Va
Thin-cap atheroma (thin)	15	Type Va
Ruptured plaques (ruptured)	17	Type VI

AHA Classification indicates American Heart Association histological criteria.[30,31]

In brief, 152 coronary artery segments were obtained at autopsy from 71 patients, including 17 consecutive patients who experienced fatal ACS without percutaneous coronary intervention and 54 consecutive patients with noncardiac death. The major coronary arteries and their branches were cut transversely at ≈5-mm intervals, and 17 ruptured plaques were detected in the 17 ACS patients (ruptured: AHA type VI, n=17). The remaining 33 patients with noncardiac death and the 17 ACS patients also had advanced unruptured plaques (≥75% cross-sectional luminal narrowing), and we assessed each segment at the narrowest point (n=114). The advanced atherosclerotic unruptured plaques were additionally divided into fibrous plaques (fibrous: fibrocellular tissue was the predominant component, and the lipid core was inconspicuous or absent; AHA type Vc, n=48), thick-cap atheroma (thick: a lipid core covered by a fibrous cap >65-μm thick; AHA type Va, n=51), and thin-cap atheroma (thin: a lipid-rich core covered by a fibrous cap <65-μm thick[32]; AHA type Va, n=15). Another 21 patients with noncardiac death who had no advanced unruptured plaques and normal coronary arteries that only showed diffuse intimal thickening (normal: AHA type I, n=21) were used as a control group.

We performed a morphological analysis of multiple lesions (n=152) obtained at autopsy from 71 patients (Figure 1). The supplementary analyses included pairwise comparison of unruptured and ruptured plaques from each heart of each patient with ACS (Data Supplement Figure I) and investigation of the correlation between traditional cardiovascular risk factors and ER stress (Data Supplement Table II and Figure III). These supplementary analyses were based on representative data from each patient. (Details of the methods used to perform the supplementary analyses are included in the expanded Methods section in the Data Supplement.)

Forty DCA specimens were obtained from 40 patients who were treated for stable angina pectoris (SAP; n=20) or unstable angina pectoris (UAP; n=20). One DCA specimen was obtained per patient, and these specimens were classified on the basis of the clinical situation at the time of DCA (Table 2). These specimens were fixed in 4% paraformaldehyde for 6 hours at 4°C and then embedded in paraffin.

Immunohistochemistry

Serial sections were examined by immunohistochemistry, as described previously.[17] In brief, sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 30 minutes. For some antibodies, antigen retrieval was performed as specified below. After blocking with 3% normal bovine serum albumin, sections were incubated with the primary antibody overnight at 4°C. KDEL (Lys-Asp-Glu-Leu) antibody, which recognizes both GRP78 and GRP94, was purchased from Stressgen (San Diego, Calif) and was used at a dilution of 1:2000. Anti-CHOP antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif) and was applied at a dilution of 1:600

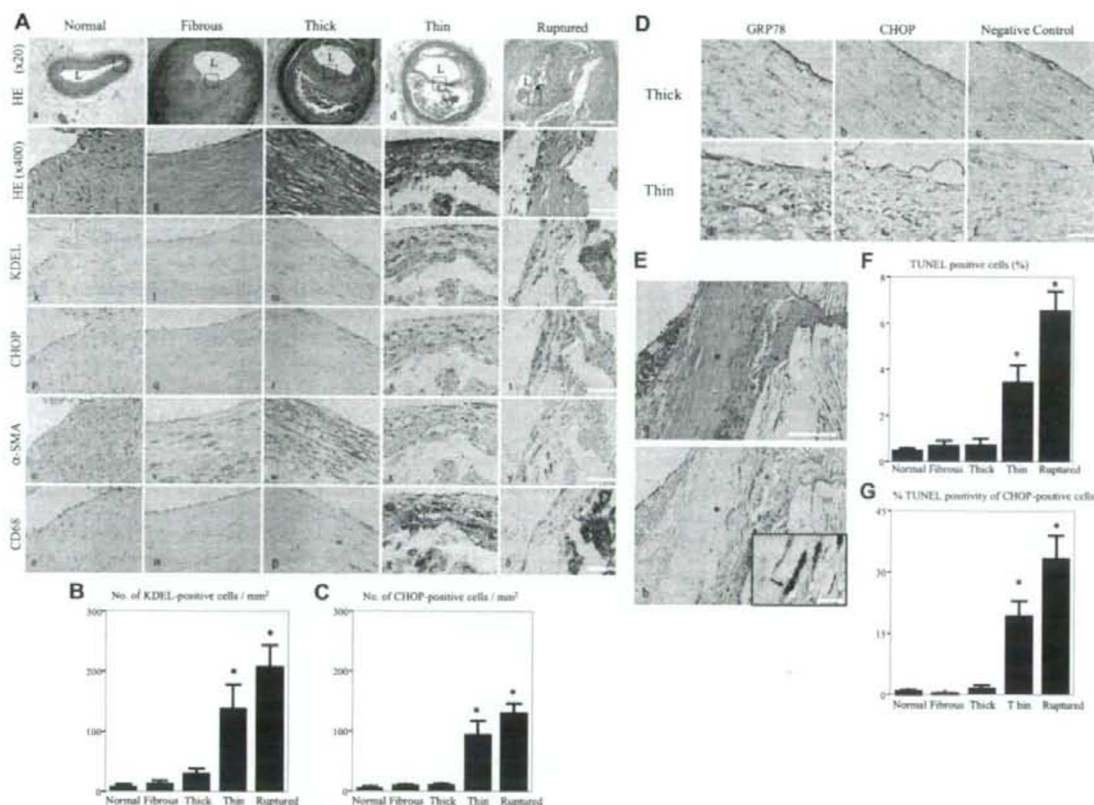

Figure 1. Induction of ER chaperones and death signals in coronary artery plaques obtained at autopsy. A, Comparison of hematoxylin-eosin (HE) staining, KDEL immunostaining, and CHOP immunostaining of normal arteries (n=14), fibrous plaques (n=48), thick-cap atheroma (n=51), thin-cap atheroma (n=15), and ruptured plaques (n=17) obtained at autopsy from 71 patients. Representative HE-stained low-power micrographs from each group (a through e). L indicates the lumen, and the arrow shows the site of plaque rupture. The parts of the intima (a) and fibrous cap (b through e) indicated by boxes are shown at a higher magnification in panels f through j. Panels k through o show KDEL immunostaining. Panels p through t show CHOP immunostaining. Panels u through y show α-smooth muscle actin (α-SMA) immunostaining. Panels z through δ show CD68 immunostaining. B and C, The number of KDEL-positive (B) and CHOP-positive (C) cells. The absolute number per square millimeter is shown for the media of a normal artery and for the fibrous caps of fibrous plaques, thick-cap atheroma, thin-cap atheroma, and ruptured plaques (B, C). D, ISH analysis of GRP78 (a, d), CHOP (b, e), and negative control (c, f) mRNA expression in thick- and thin-cap atheromas. E, Comparison of HE staining (a) with double immunostaining (b). Colocalization of CHOP (red) with TUNEL-positive cells (brown) in the cap of a ruptured plaque. The area indicated by asterisks is shown at a higher magnification in the inset. Arrows show CHOP and TUNEL double-positive cells. F and G, Percentage of TUNEL-positive cells (F) and percentage of TUNEL-positive cells among CHOP-positive cells (G) in the fibrous cap. Scale bars represent 1 mm (A, a through e), 50 μm (A, f through δ, D, and E), and 20 μm (E, in inset). *$P<0.05$ vs normal plaque.

after antigen retrieval by incubation for 10 minutes at room temperature in 5 μg/mL proteinase K. Anti-phospho-c-JUN NH_2-terminal kinase antibody was used to detect c-JUN kinase (JNK), which is involved in the UPR.[16] It was obtained from Cell Signaling (Danvers, Mass) and was applied at a dilution of 1:100 after heat retrieval for 15 minutes at a sub-boiling temperature in 1 mmol/L EDTA (pH 8.0). Colon carcinoma sections were stained with anti-phospho-JNK antibody as a positive control. Anti-α-smooth muscle actin antibody and anti-human CD68 antibody (DAKO, Glostrup, Denmark) were used to identify SMCs and macrophages, respectively, and were used

TABLE 2. Human Coronary Specimens (Atherectomy; n=40)

Origin and Classification of Plaques	No. of Specimens
SAP	20
UAP	20

at a dilution of 1:200. The EnVision kit (DAKO) was then used for immunostaining. Application of the KDEL antibody or the CHOP antibody after preincubation with each synthetic peptide used for immunization (KDEL: synthetic peptide SEKDEL, 10 μg/mL, Tore Bio, CHOP peptide: 10 μg/mL, Santa Cruz Biotechnology) resulted in no detectable signals, demonstrating the specificity of the antibody (Data Supplement Figure II).

Terminal dUTP Nick End-Labeling Method and Double Immunohistochemistry

Cells undergoing apoptosis were identified by the terminal dUTP nick end-labeling (TUNEL) method with the ApopTag In Situ Apoptosis Detection Kit (Chemicon, Temecula, Calif), as described previously.[8] For simultaneous identification of CHOP and TUNEL immunoreactivity, double immunostaining of specimens was performed. First, the TUNEL method was performed with an ApopTag

kit, and then CHOP was detected with an alkaline phosphatase-labeled secondary antibody with NewFukusin (DAKO).

In Situ Hybridization

Digoxigenin-labeled cRNA probes and the negative control (LNE120) were purchased from Direct Communications Inc (Hirosaki, Japan), and the sequences were as follows: GRP78: 5'-UGGAAUUCGAGUCGAGCCACCAACAAGAACAAUUU-CAUCAAUAUCAGACUUCUUCAAAUCAGAAUCUUCCAAC-ACUUUCUGGACGGGCUUCAUAGUAGACCGGAACAGAU-CCA UGUUGAG-3'; CHOP: 5'-AUGCUCCCAAUUGUUCAUG-CUUGGUGCAGAUUCACCAUUCGGUCAAUCAGAGCUCGG-CGAGUCGCCUCUACUUCCCUGGUCAGGCGCUCGAUUUC-CUGCUUGAGCCGUUCAUUCUCUUC-3'. In situ hybridization (ISH) was performed as described previously[33] with a Microprobe manual staining system (Fisher Scientific, Pittsburgh, Pa). In brief, hybridization of the probes (1 μg/mL) was performed for 120 minutes at 50°C, and then anti-digoxigenin-Ap (x250, Roche, Basel, Switzerland), as the secondary antibody, and NBT/BCIP stock solution (×50, Roche) were added.

7-KC Staining

Snap-frozen samples were obtained from 12 patients, comprising 6 with thick-cap atheroma and 6 with thin-cap atheroma. Frozen sections were fixed in 10% neutral-buffered formalin for 1 hour at room temperature. After blocking with 3% normal bovine serum albumin, the sections were incubated overnight at 4°C with anti-7-KC antibody (Nikken Seil Corporation, Fukuroi, Japan) at a dilution of 1:100, followed by incubation with an EnVision kit for 30 minutes.

Statistical Analysis

Data are expressed as mean±SEM. For the autopsy study of multiple lesions from many patients (Figure 1), statistical analysis was performed with the Kruskal–Wallis H test and a post hoc Mann-Whitney U test. For the DCA specimens, statistical analysis was performed with the Mann-Whitney U test. Experiments with cultured cells were performed at least 3 times each. Data obtained with cultured cells were analyzed statistically by the unpaired Student t test or ANOVA, followed by the Bonferroni test. Comparison of categorical variables was done with Fisher exact test. In all analyses, $P<0.05$ was accepted as statistically significant. The expanded Methods section, covering supplementary data and in vitro studies is included as an online-only Data Supplement.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Upregulation of ER Chaperones and Apoptosis in the Fibrous Caps of Thin-Cap Atheroma and Ruptured Plaques

In the fibrous caps of thin-cap atheroma and ruptured plaques, KDEL and CHOP immunostaining showed a marked increase compared with the level of staining in the fibrous caps of thick-cap atheroma and fibrous plaques (Figure 1A k through t, Figure 1B, and Figure 1C). KDEL-positive cells were more numerous than CHOP-positive cells in the fibrous caps of thin-cap atheroma and ruptured plaques. Most of the CHOP-positive cells also expressed KDEL, as shown by staining of serial sections. In the same hearts of the ACS patients, there was a significant difference of KDEL- and CHOP-positive cells between the unruptured and ruptured plaques (Data Supplement Figure I). We also assessed ER chaperone (GRP78) and CHOP expression at the mRNA level by ISH (Figure 1D). Furthermore, we confirmed that the KDEL- and CHOP-positive cells were SMCs or macrophages by immunostaining of serial sections with anti α-smooth muscle actin and anti-CD68, respectively (Figure 1A, u through δ). In the fibrous caps of thin-cap atheroma and ruptured plaques, colocalization of CHOP immunoreactivity with TUNEL-positive cells was observed by double immunostaining (Figure 1E). The percentage of TUNEL-positive cells (Figure 1F) and the percentage of TUNEL-positive cells among CHOP-positive cells (Figure 1G) were increased compared with the findings in other specimens. Immunostaining for phospho-JNK, which is a proapoptotic factor involved in ER stress, revealed no immunoreactivity in the fibrous caps of thick, thin, or ruptured plaques (data not shown). In normal coronary artery specimens with diffuse intimal thickening (Figure 1A, k and p), there was no KDEL or CHOP positivity. In the region around the necrotic core of advanced plaques, KDEL positivity was only observed in macrophages. There was no significant difference in the number of KDEL-positive cells within the area surrounding the necrotic core of thick-cap atheromas ($726\pm88/mm^2$), thin-cap atheromas ($741\pm52/mm^2$), and ruptured plaques ($651\pm102/mm^2$).

Upregulation of ER Stress in Atherectomy Specimens From Patients With UAP

To estimate the activation of ER stress related to the clinical situation, we examined histological sections obtained at DCA. Morphometric analysis demonstrated that the number of KDEL- and CHOP-positive cells was significantly higher in patients with UAP than in patients with SAP ($P<0.05$; Figures 2A, 2B, and 2C). The KDEL- and CHOP-positive cells were confirmed to be SMCs and macrophages (Figure 2A). When ER chaperone (GRP78) and CHOP mRNA levels were analyzed by quantitative reverse-transcription polymerase chain reaction or ISH, GRP78 expression was increased in patients with UAP ($P=0.14$; Figure 2D), whereas CHOP expression was significantly higher in UAP patients than in SAP patients ($P<0.05$; Figure 2E). On the other hand, both GRP78 and CHOP were significantly increased according to ISH (Figure 2F), but we could not confirm a significant increase of GRP78 by reverse-transcription polymerase chain reaction. This may have been because the number of fresh DCA specimens was too low.

Immunohistochemical Detection of 7-KC in the Fibrous Caps of Atherosclerotic Plaques

To explore the likely molecular mechanism of activation of ER stress and the mechanistic link to apoptosis, we investigated plaque lipids by staining frozen coronary artery sections with anti-7-KC antibody (Figure 3), and the in vitro studies were performed (Figure 4). Immunoreactivity for 7-KC was increased in the fibrous caps of thin-cap atheroma, whereas no immunoreactivity was detected in the fibrous caps of thick-cap atheroma (Figure 3). In the region around the lipid core, however, 7-KC immunoreactivity was visible in both types of atheroma (Figure 3).

Upregulation of ER Chaperones, CHOP, and Apoptosis by 7-KC and Effects of CHOP Knockdown by siRNA in CASMCs or THP-1 Cells

Exposure of cells to 7-KC increased the expression of GRP78 and CHOP mRNA, whereas this increase was prevented by

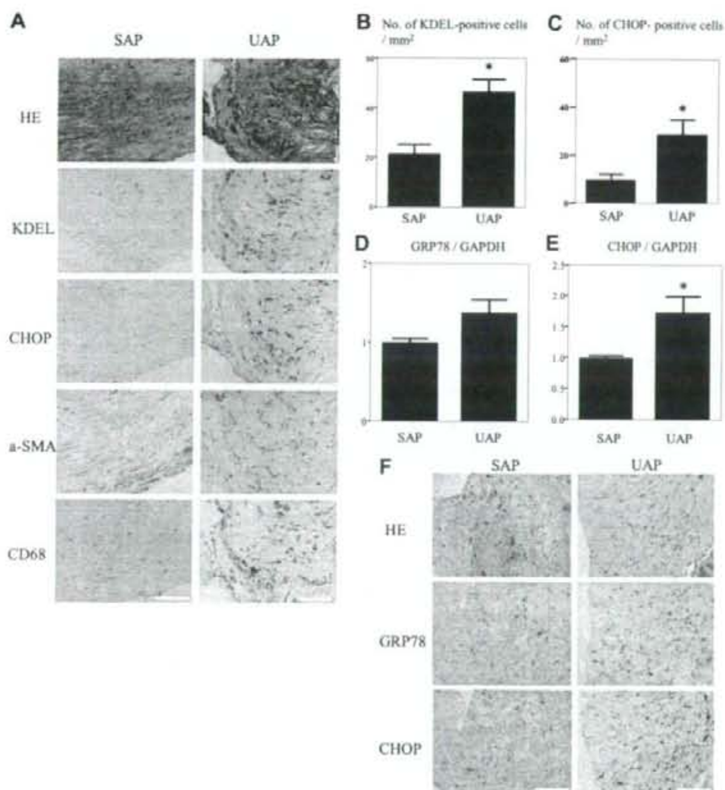

Figure 2. Induction of ER chaperones and death signals in atherectomy specimens obtained from the culprit lesions of patients with UAP. A, Comparison of hematoxylin-eosin (HE) staining with KDEL, CHOP, α-smooth muscle actin (a-SMA), and CD68 immunostaining of the serial sections of 32 atherectomy specimens obtained from patients with SAP (n=16) or UAP (n=16). B and C, Number of KDEL-positive (B) and CHOP-positive (C) cells per square millimeter. D and E, Comparison of GRP78 and CHOP expression normalized for GAPDH in 8 specimens from patients with SAP (n=4) or UAP (n=4) by quantitative reverse-transcription polymerase chain reaction. F, HE staining and ISH analysis of GRP78 (a, d) and CHOP (b, e) mRNA expression in specimens from SAP patients (n=6) or UAP patients (n=6). Scale bar represents 50 μm. *$P<0.05$ vs SAP.

the antioxidants *N*-acetylcysteine or glutathione (Figure 4A). We observed intracellular production of reactive oxygen species after exposure to 7-KC, whereas glutathione reduced reactive oxygen species production (Figure 4B). We also

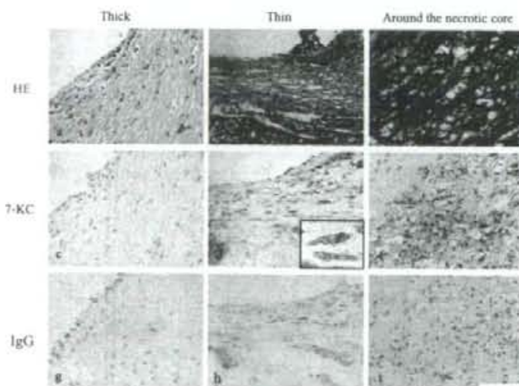

Figure 3. Immunohistochemical detection of 7-KC in the fibrous cap of thin-cap atheroma. Comparison of hematoxylin-eosin (HE) staining with 7-KC or IgG immunostaining in the fibrous caps of thick-cap atheroma (n=6) and thin-cap atheroma (n=6), as well as around the necrotic core of thick-cap atheroma. 7-KC immunostaining is shown at a higher magnification in the inset. Scale bar represents 50 μm.

examined the effects of 7-KC on apoptosis of CASMCs and THP-1 cells (Figure 4C). Treatment with 7-KC increased FITC-annexin and propidium iodide staining in a dose-dependent (Figure 4C, b and c) and time-dependent (data not shown) manner. Treatment of CASMCs and THP-1 cells with 7-KC for 24 hours also induced apoptosis along with the induction of ER chaperones and CHOP at the protein level (Figure 4D). When CASMCs and THP-1 cells were simultaneously incubated with 7-KC and *N*-acetylcysteine or glutathione, both antioxidants reduced the induction of ER chaperones (Figure 4D). Quantitative analysis revealed that most of the CHOP-positive cells coexpressed KDEL (88.2% of CHOP-positive CASMCs and 72.7% of CHOP-positive THP-1 cells; $P<0.05$, Fisher's exact test), whereas there were few KDEL-negative and CHOP-positive cells, which suggests that CHOP was involved in the mediation of ER-initiated signaling (Figure 4D, c and d). Treatment of THP-1 cells with 7-KC induced CHOP, whereas 2 different siRNAs targeting CHOP caused the knockdown of CHOP expression (Figure 4E, a). Knockdown of CHOP expression by siRNA decreased the number of TUNEL-positive THP-1 cells after exposure to 7-KC (Figure 4E, b and d). Similarly, the knockdown of CHOP expression by siRNA decreased the number of TUNEL-positive CASMCs after exposure to 7-KC (Figure 4E, c).

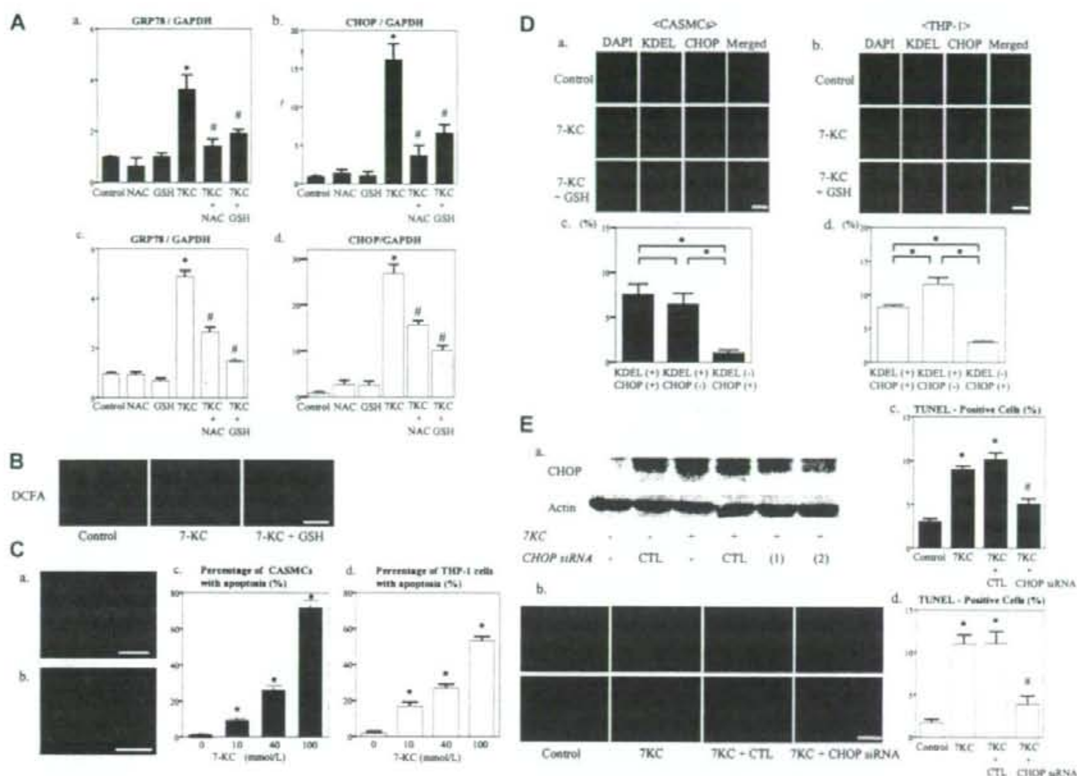

Figure 4. Upregulation of ER chaperones, CHOP, and apoptosis by exposure to 7-KC and effect of CHOP knockdown by siRNA in cultured CASMCs or THP-1 cells. A, Comparison of GRP78 and CHOP expression normalized for GAPDH by quantitative reverse-transcription polymerase chain reaction. CASMCs or THP-1 cells were incubated with 7-KC (80 mmol/L) in the absence or presence of *N*-acetylcysteine (NAC) or glutathione (GSH) for 12 hours. B, Measurement of reactive oxygen species (ROS) generation after exposure to 7-KC and 2', 7'-dichlorofluorescin diacetate (DCFH-DA) in the absence or presence of GSH for 12 hours. C, FITC-annexin V and propidium iodide staining for apoptosis of CASMCs and THP-1 cells incubated with 7-KC (a). Exposure to 7-KC induced apoptosis of CASMCs (b) and THP-1 cells (c) in a dose-dependent manner. D, KDEL and CHOP staining of CASMCs (a) and THP-1 cells (b) after incubation with 7-KC in the absence or presence of GSH for 24 hours; c and d, quantitative analysis of immunohistochemical staining of CASMCs (c) and THP-1 cells (d). E, Western blotting for CHOP after exposure to 7-KC with or without CHOP siRNA (a). TUNEL staining of THP-1 cells (b) and quantitative analysis of TUNEL-positive CASMCs (c) and THP-1 cells (d). CTL indicates the nonsilenced control. Scale bars represent 50 μm (B, C, and E) and 20 μm (D). *P<0.05 vs control (A, C, and E). #P<0.05 vs treatment with 7-KC (E). Experiments were performed at least 3 times. The data are expressed as mean±SEM. The immunofluorescent staining and Western blotting data are representative of at least 3 independent experiments.

Discussion

The present study revealed a marked increase of ER chaperone expression, CHOP expression, and apoptosis in the fibrous caps of thin-cap atheroma or ruptured plaques, as well as in atherectomy specimens from UAP patients, which suggests that ER stress may play a role in the progression of plaque vulnerability and the occurrence of acute complications of coronary atherosclerosis in humans. Because of the inherent limitations of an autopsy study, we could not exclude the possibility that UPR activation occurred after plaque rupture. Previous reports have shown that ER chaperones, such as GRP78 or GRP94, may have a protective effect against ischemia/reperfusion injury.[34] However, the presence of apoptotic changes in the thin-cap atheroma of the patients with noncardiac death suggested that the findings we observed in ruptured plaques represented the evolution of such changes in the thin-cap atheroma rather than being secondary to plaque rupture or ischemia/reperfusion injury. Only specimens from patients without percutaneous coronary intervention were studied, to exclude the influence of this intervention. We also observed an increase of ER stress-related changes in freshly fixed atherectomy specimens obtained from UAP patients compared with those from SAP patients. This suggests that ER stress activation was related to the clinical situation, and the autopsy specimens were only slightly affected by postmortem protein degradation.

Among the oxysterols, 7-KC is most frequently detected at high levels in atherosclerotic plaques and in the plasma of patients with a high cardiovascular risk.[26,35] To the best of our knowledge, however, 7-KC has not previously been detected in human atherosclerotic coronary artery sections by immunohistochemistry. It has been reported that 7-KC induces the

production of reactive oxygen species, activation of the UPR, and induction of apoptotic death in cultured human SMCs.[28] We demonstrated that the fibrous caps of thin-cap atheroma were immunohistochemically positive for 7-KC, a finding consistent with the increase of ER stress/UPR markers.

Treatment of CASMCs with 7-KC induced ER stress and activation of the UPR, findings that were consistent with the results of a previous study on aortic SMCs,[28] and these changes also occurred in THP-1 cells. This 7-KC–induced cellular damage was prevented by antioxidants (*N*-acetylcysteine and glutathione), which was also consistent with a previous report.[36] Accordingly, the present findings suggest that an increase of ER stress due to 7-KC induces apoptosis of SMCs and macrophages through the production of reactive oxygen species.

ER stress induces apoptosis via the CHOP-, JNK-, and caspase-12–dependent signaling pathways.[16] CHOP is mainly induced at the transcriptional level by ER stress,[12,37] after which its overexpression leads to apoptosis.[11,16,38] CHOP knockout mice show normal development and normal fertility but exhibit less apoptosis in response to ER stress.[16,21] Thus, detection of the induction of CHOP indicates an increase of ER-initiated apoptosis. Although the direct transcriptional target of CHOP has not been found,[39] the Bcl-2 pathway may be involved in the downstream connection between CHOP and apoptosis.[28,39] Caspase-12 is only activated by ER stress.[13,16,18] Although caspase-12 has been cloned in mice and rats, it is not yet possible to explore the role of this caspase in humans.[40] JNK is 1 of the stress-activated protein kinases that has been shown to induce apoptosis in response to ER stress.[13,16] We demonstrated that TUNEL-positive SMCs and macrophages were significantly increased in the fibrous cap, with CHOP (but not JNK) being induced simultaneously. Treatment of CASMCs or THP-1 cells with 7-KC induced CHOP, whereas knockdown of CHOP expression by siRNA led to a decrease of TUNEL-positive cells after exposure to 7-KC. Because CHOP is a transcription factor that specifically mediates ER-initiated apoptosis, the induction of CHOP in ruptured and unstable plaques supports the activation of ER-initiated apoptosis. However, our autopsy study could not exclude the possibility that the cells underwent apoptosis independently of CHOP, whereas the TUNEL assay gave false-positive results in the clinical specimens.

Unfortunately, we could not confirm whether or not the relationship between thinning of the fibrous cap and ER stress was causative because of the lack of a suitable animal model of plaque rupture. On the other hand, together with the present finding that 7-KC induced ER stress, the possibility that ER stress causes plaque vulnerability is also supported by the following reports. In cultured peritoneal macrophages, excessive accumulation of free cholesterol has been found to initiate ER stress, increase CHOP expression, and increase apoptosis.[21] Moreover, in vivo studies with $apoE^{-/-}$ mice have shown that lesional necrosis can be diminished by a decrease in the cholesterol level.[22] In addition, the present study demonstrated that expression of ER chaperones was upregulated to a similar extent in macrophages surrounding the necrotic cores of thick-cap atheroma, thin-cap atheroma, and ruptured plaques, which suggests that ER stress may contribute to the progression of plaque vulnerability by inducing macrophage apoptosis.

In conclusion, the present findings support the possibility that ER stress and/or the UPR induces apoptosis of SMCs and macrophages, thus increasing the vulnerability of coronary artery plaques, which may lead to ACS and a fatal outcome in patients with coronary artery disease.

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Disclosures

None.

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