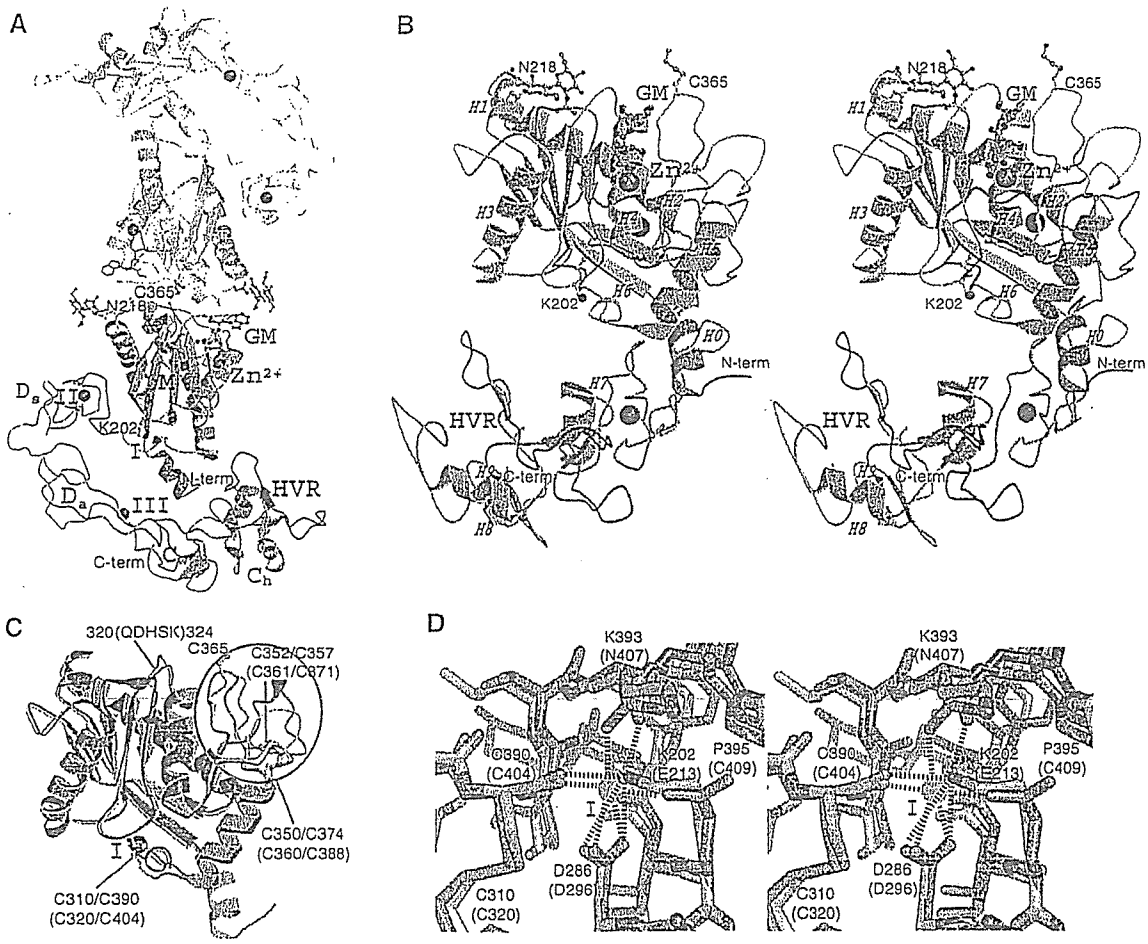

Figure 1 MDC architecture. (A) VAP1 dimer viewed from the NCS axis. The H0-helix, M-domain, linker, D_s-, D_a-, C_w-, and C_h-domains and HVRs belonging to the one monomer are shown in red, yellow, gray, cyan, pink, gray, green and blue, respectively. The disulfide-linked counterpart is shown in gray. Zinc and calcium ions are represented as red and black spheres, respectively. The NAG (*N*-acetyl-glucosamine, in orange) moieties linked to Asn218, the calcium-mimetic Lys202 and the bound inhibitor GM6001 (GM, in green) are in ball-stick representations. (B) Stereo view of VAP1 monomer from the direction nearly perpendicular to (A). The helix numbers are labelled. (C) Superposition of the M-domains of ADAM33 (blue) and VAP1 (yellow). The calcium ion bound to site I and the zinc ion in ADAM33 are represented by black and red spheres, respectively. The disulfide bridges are indicated in black and blue letters for VAP1 and ADAM33, respectively. The QDHSK sequence for the dimer interface in VAP1 (residues 320–324) is in red. (D) Comparison of the calcium-binding site I structures of ADAM33 (blue) and VAP1 (yellow) *in stereo*. The residues in ADAM33 and in VAP1 are labelled in blue and black, respectively. A calcium ion and a water molecule bound to ADAM33 are represented as green and red spheres, respectively. The ammonium group of Lys202 in VAP1 occupies the position of the calcium ion in ADAM33. In ADAM33 (Orth *et al*, 2004), side-chain oxygen atoms of Glu213, Asp296 and Asn407, the carbonyl oxygen of Cys404 and a water molecule form the corners of a pentagonal bipyramid and ligand to the calcium ion.

and faces toward the catalytic site in the M-domain. The C-terminus Tyr610 is located proximal to the boundary between the D_a- and C-domains (Figure 1A and B). Aside from Cys365, each monomer contains 34 cysteinyl residues, all of which are involved in disulfide bonding, and their spacings are strictly conserved among ADAMs (Figure 2 and Supplementary Figure 1) except within the substrate-binding (between the helices H4 and H5) and the HVR (see below) regions. Figure 2 provides a selected subset of the sequence alignments and the entire alignments of VAP1 and 39 ADAM sequences, including all 23 human ADAMs so far available, can be found as Supplementary Figure 1.

M-domain

Each VAP1 M-domain corresponds to a very similar structure to that of ADAM33 (Orth *et al*, 2004), with a flat ellipsoidal shape having a central core made up of five stranded β-sheets and five α-helices and a conserved methionine (Met-turn) below the active site histidine residues, which bears the typical structural feature of metzincin family of metalloproteinases (Bode *et al*, 1993). However, they differ in the dimer interface and the loop structure around the substrate-binding site (Figure 1C) that corresponds to the variable region in the primary structure (between the helices H4 and H5, see Figure 2). The N-terminal helix (H0) is also unique in VAP1. The dimer interface is best characterized by the recognition sequence QDHSK (residues 320–324, see Figure 1C and Supplementary Figure 2A–C) and by Cys365, however these are not conserved among ADAMs; therefore, none of the ADAMs' M-domains are suggested to form a stable dimer as VAP1. A peptide-like hydroxamate inhibitor GM6001 binds to VAP1 (Figure 1A and B, and Supplementary Figure 2D and E) in exactly the same manner as in the marimastat-ADAM33 M-domain complex (Orth *et al*, 2004), suggesting that the catalytic sites of VAP1 and ADAM33 share a common substrate recognition mechanism. The ADAM33

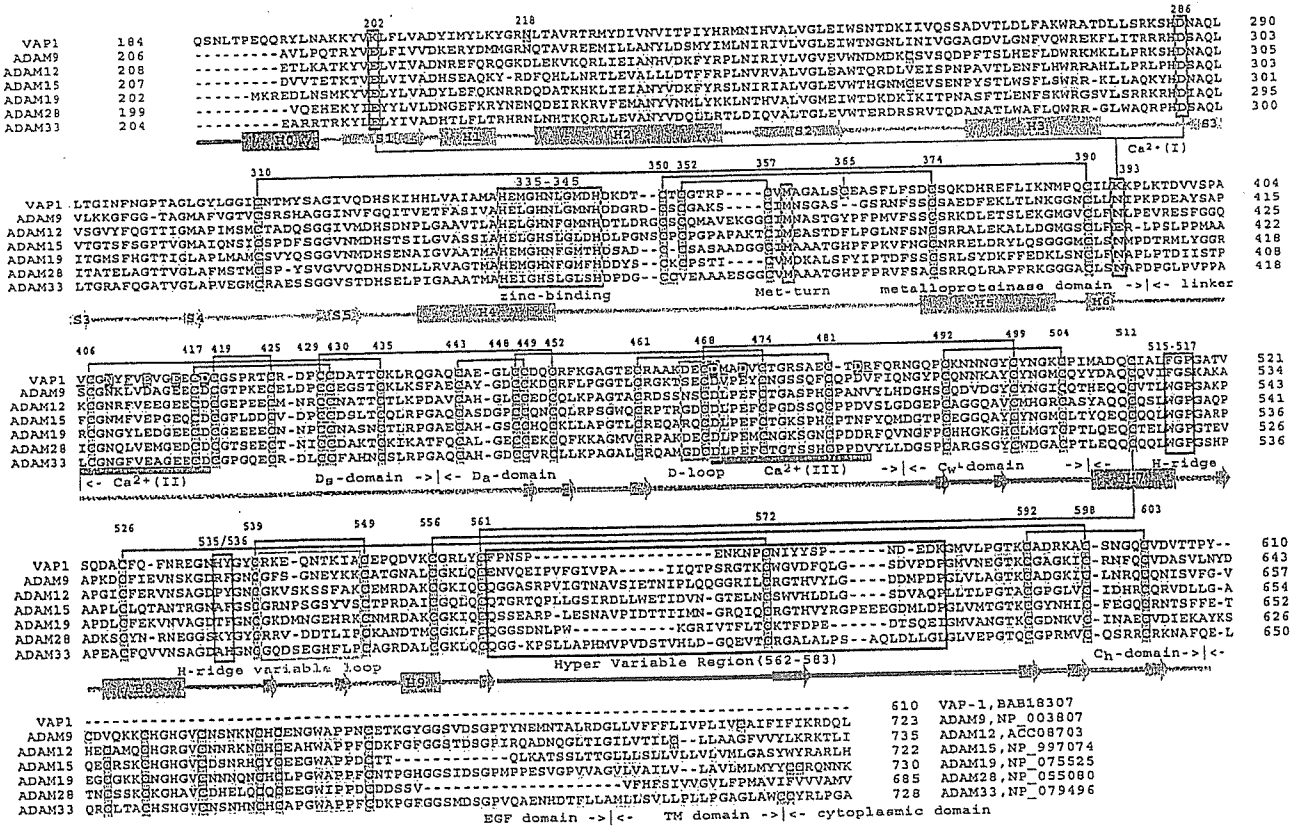

Figure 2 Sequence alignments of VAP1 and human ADAMs. The cysteinyl residues and the conserved residues are shaded in pink and yellow, respectively. Disulfide bridges, secondary structures and domains are drawn schematically. The HVR, calcium-binding site I, catalytic site and disintegrin-loop (D-loop) are boxed in blue, red, green and cyan, respectively. The hydrophobic ridges (H-ridges) are indicated. Calcium-binding sites II and III and the coordinating residues (shaded in red) are indicated. The NCBI accession numbers for the sequences are indicated.

M-domain structure suggests that most ADAMs have a Ca^{2+}-binding site (designated Ca^{2+}-binding site I) opposing the active-site cleft; however, in VAP1, the distal ammonium group of Lys202 substitutes for the Ca^{2+} ion (Figure 1D). Replacement of the calcium-coordinating glutamate residue with lysine also occurs in ADAM16, ADAM25 and ADAMs38–40 (Supplementary Figure 1).

C-shaped arm

The D-domain follows the M-domain, with a short linker that allows slightly variable domain orientations at V405 as a pivotal point (Figure 3C). The D-domain is further divided into two structural subdomains (Figure 3), the 'shoulder' (D_s-domain, residues 396–440) and the 'arm' (D_a-domain, residues 441–487). The D_s- and D_a-domains constitute a continuous C-shaped arm, together with the following N-terminus region of the C-domain which we designate the 'wrist' (C_w-domain, residues 488–505). There are three disulfide bonds in the D_s-domain, three in the D_a-domain and one in the C_w-domain. The subdomains are connected by single disulfide bridges (Figures 2 and 3A) with slightly variable angles (Figure 3B).

Both the D_s- and D_a-domains contain structural calcium-binding sites. In the D_s-domain, the side-chain oxygen atoms in residues Asn408, Glu412, Glu415 and Asp418, and the carbonyl oxygen atoms of Val405 and Phe410 are involved in pentagonal bipyramidal coordination and constitute Ca^{2+}-binding site II (Figures 2 and 3A). Notably, these residues are strictly conserved among all known ADAMs (Supplementary Figure 1). However, the side-chain oxygens of Asp469, Asp472 and Asp483, and carbonyl oxygens of Met470 and Arg484 form the corners of a pentagonal bipyramid to the calcium ligand and constitute the D_a-domain Ca^{2+}-binding site III (Figures 2 and 3A) and these residues are highly conserved among ADAMs except ADAM10 and ADAM17 (Supplementary Figure 1). Because of the few secondary-structural elements, bound calcium ions and the disulfide bridges are essential for the structural rigidity of ADAM's C-shaped arm. The RGD-containing disintegrin trimestatin (Fujii *et al*, 2003) has a similar structure with the D_a-domain (r.m.s.d of 1.24 Å, Figure 3B); however, no disintegrins have been shown to bind Ca^{2+} ions.

Using isolated D-domains or portions thereof, numerous ADAMs and P-III SVMPs have been shown to interact specifically with particular integrins (Evans, 2001; White, 2003; Calvete *et al*, 2005). However, the disintegrin-loop is packed against the C_w-domain and a disulfide bridge (Cys468–Cys499) further stabilizes the continuous structure (Figure 3A). Therefore, the disintegrin-loop is inaccessible for protein binding.

Hand domain

The 'hand' domain (C_h-domain, residues 505–610) follows the C_w-domain. The C_h-domain, together with the C_w-domain, constitutes a novel fold (Figure 4A). In either crystal form, VAP1 dimers interact with molecules of neighboring

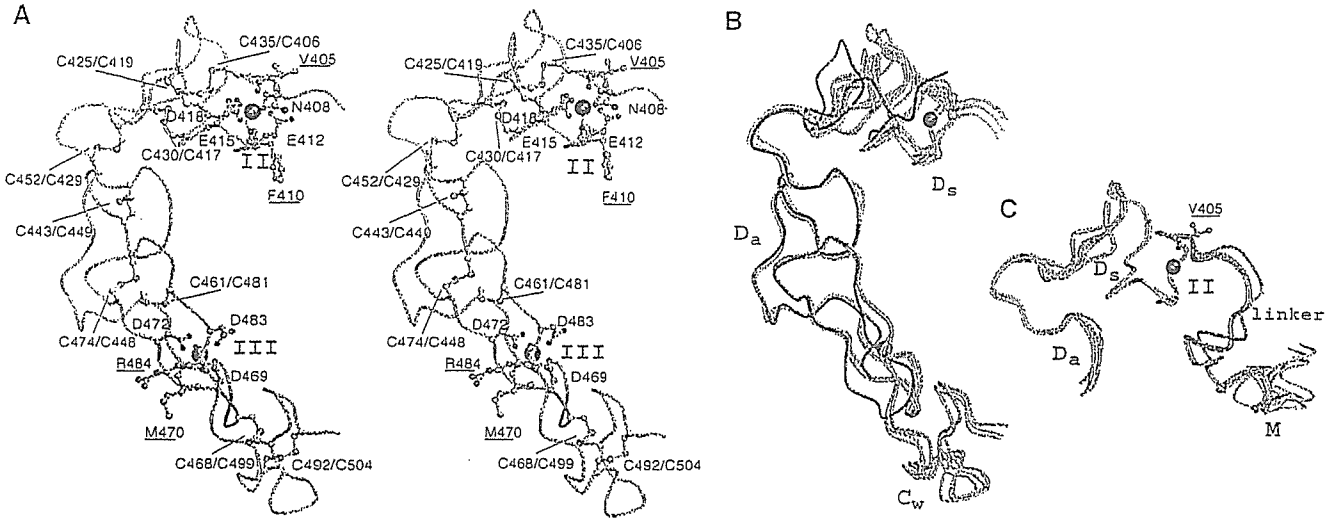

Figure 3 Arm structure. (A) Arm structure *in stereo*. The D_s-, D_a-, and C_w-domains are in cyan, pink and light green, respectively. The calcium-coordinating residues and the disulfide bridges are shown in red and green, respectively. The residues with carbonyl oxygen atoms involved in calcium coordination are underlined. Calcium ions are represented as black spheres. The disintegrin-loop (DECD) is in blue. (B) Superimposition of the four D_a-domains of VAP1 and trimestatin (1J2L). Trimestatin and its RGD loop are shown in red and blue, respectively. (C) Superimposition of the four D_s-domains. The linker between the M- and D_s-domains is shown in gray. Val405 at the pivotal point is indicated.

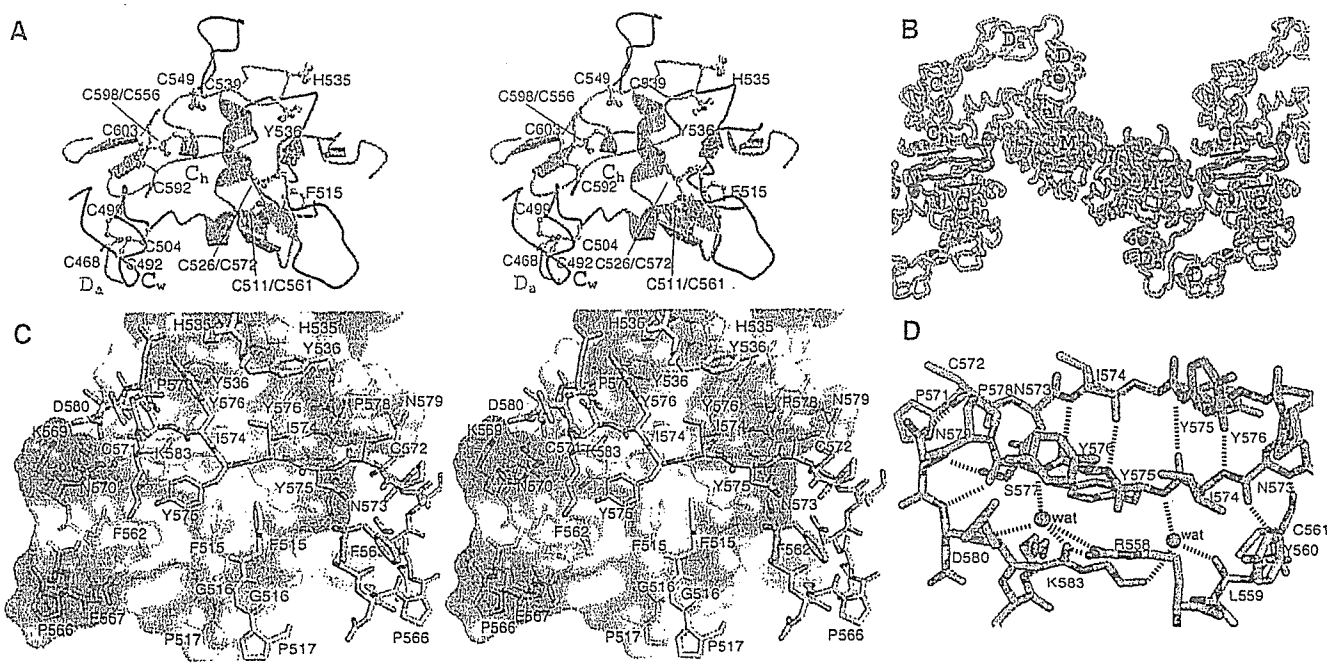

Figure 4 C-domain architecture and HVR. (A) The C-domain architecture *in stereo*. The C_w- and C_h-domains are in gray and light green, respectively. The disulfide bridges and the residues forming the hydrophobic ridges are indicated. The HVR and its NCS counterpart are shown in red and blue, respectively. The variable loop (residues 539–549), flanked by two adjacent cysteine residues, is in green. (B) Crystal packing in the orthorhombic crystal. The crystallographically equivalent molecules (HVRs) are in cyan (blue) and pink (red), respectively. The arrows indicate the directions of the HVR chains. Zinc and calcium ions are represented as red and black spheres, respectively. (C) Interactions between the HVRs (cyan and pink) *in stereo*. The molecular surface of the cyan molecule is shown with the electrochemical surface potential (red to blue). The residues constituting the hydrophobic ridges are in yellow. The residues are labelled in blue and red for cyan and pink, respectively. (D) Water-mediated hydrogen-bond network in the HVR. The HVR residues are in pink and cyan; non-HVR residues in the pink molecule are in gray.

units through the C_h-domains such that the molecules form a handshake (Figure 4B). Each C_h-domain interacts with its counterpart through a relatively large complementary surface of 860 Å^2 forming another NCS at the center, although VAP1 exists as dimers, not as oligomers, and is mono-dispersed in solution (data not shown).

HVR as a potential adhesive interface

C_h-domain residues 562–583 are predominantly involved in the handshake (Figure 4B). This is the region in which the ADAM sequences are most divergent and variable in length (16–55 aa) (Figure 2 and Supplementary Figure 1). We have designated this as the hyper-variable region (HVR). The HVR

is subdivided into two structural elements. The N-terminal portion (residues 562–572) fits into an extended loop, filling the gap between the M-domain and the neighboring molecule's C_h-domain and thus fixing the position of the arm (Figure 4B). The variable structures and less-specific interactions suggest that this loop is stabilized by crystal packing. Some ADAMs possess a putative fusion peptide in this segment typical of viral fusion proteins (Blobel *et al*, 1992; Yagami-Hiromasa *et al*, 1995), although their role in the actual fusion process has not been demonstrated. However, the remainder of the HVR (residues 572–583) interacts extensively with its counterpart by forming an antiparallel β strand at the center (Figure 4C and D). Although the ability to form β strand is predictable from the sequence, this β strand is stabilized mainly by interchain interactions (Figure 4D). There are no intrachain hydrogen bonds between residues 574–577 and the remainder of the C_h-domain; however a water-mediated hydrogen-bond network stabilizes this segment (Figure 4D). Therefore, it appears, that this β strand might be formed by the induced-fit mechanism upon the association of the C_h-domains and that the conserved disulfide bond (Cys526–Cys572, see Figure 4D) may stabilize the structure when the HVRs are isolated in solution. In addition to the main-chain hydrogen bonds, side-chain atoms (particularly residues I574, Y575, Y576 and P578) in the HVR β strand contribute numerous von der Waals interactions with their counterparts. Aside from the HVR, aromatic residues located at both sides of the β strand in close proximity to the NCS axis create additional interaction surfaces: residues Phe515, Gly516, His535 and Tyr536 in the loop regions form hydrophobic ridges that fit complementarily into the NCS region (Figure 4C). The hydrophobic ridges are highly conserved among ADAMs (Figure 2 and Supplementary Figure 1), thus, in part, they may also constitute binding surfaces.

Discussion

The VAP1 structures reveal highly conserved structural calcium-binding sites and the numbers and the spacings of cysteinyl residues that are essential for maintaining structural rigidity and spatial arrangement of the ADAMs' MDC domains. The C-shaped MDC architecture implies meaningful interplay between the domains and their potential roles in physiological functions.

The HVR creates a novel interaction interface in collaboration with the conserved hydrophobic ridges. Different ADAMs have distinct HVR sequences, which result in distinct surface features, thus, they may function in specifying binding proteins. The HVR is at the distal end of the C-shaped arm and points toward the M-domain catalytic site, with a distance of ~4 nm in between them. Collectively, these observations suggest that the HVR captures the target or associated protein that is processed by the catalytic site (Figure 5). The disintegrin portion is located opposit to and apart from the catalytic site and, thus, might play a primary role as a scaffold that allocates these two functional units spatially. The C-shaped structure also implies how the ADAMs' C-domains cooperate with their M-domains (Reddy *et al*, 2000; Smith *et al*, 2002). In membrane-bound ADAMs, the EGF-like domain (~60 aa) follows the C_h-domain (Figure 2) and presumably works as a rigid spacer connecting the MDC-domains with and orientating against the membrane-spanning region (Figure 5A). Many ADAMs are proteolytically inactive (because of the defects in the catalytic HEXXHXXGXXHD sequence or the post-translational removal of the M-domain), and several of these are important developmentally. Therefore, the HVR may also work to modulate cell–cell and cell–matrix interactions. There is some experimental evidence for C-domain-mediated adhesion. Peptides encompassing the HVR and the hydrophobic ridge from P-III SVMPs interfere with platelet interaction and collagen binding (Kamiguti *et al*, 2003). A recombinant atrolysin-A C-domain specifically binds collagen I and von Willebrand factor (vWF) and blocks collagen–vWF interaction (Jia *et al*, 2000; Serrano *et al*, 2005). ADAM12 interacts with cell-surface syndecan through its C-domain and mediates integrin-dependent cell spreading (Iba *et al*, 2000). The D/C-domain portion of ADAM13 binds to the ECM proteins laminin and fibronectin (Gaultier *et al*, 2002). However, most of these studies do not assign specific regions of the C-domain to these interactions and the molecular recognition mechanisms are to be elucidated.

ADAM10 and ADAM17 lack the Ca^{2+}-binding site III and show less sequence similarities in the C-domain with other

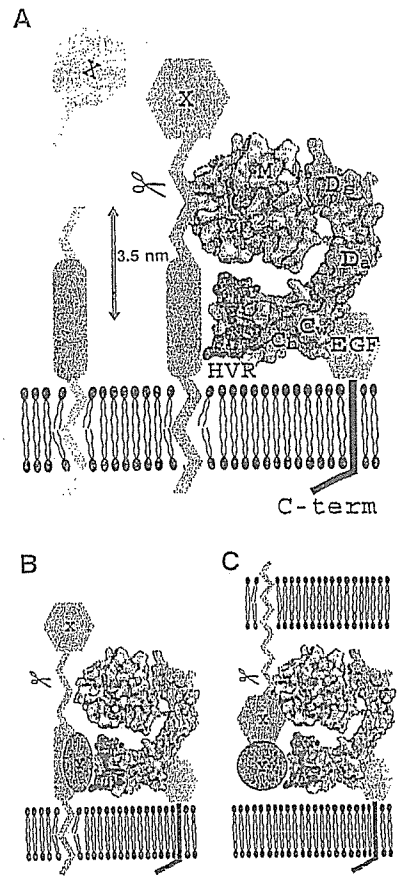

Figure 5 Models for ADAM's shedding. The molecular surface of the VAP1 monomer, without VAP1's unique H0-helix, are colored as in Figure 1A. Hydrophobic ridges are in yellow. EGF-like, transmembrane and cytoplasmic domains are represented schematically. (A) Membrane-anchored substrate molecule 'X' is directly recognized and captured by the HVR on the membrane-bound ADAM molecule. The distance between the center of the HVR (Tyr575) and the catalytic zinc ion is about 3.5 nm. (B) Substrate 'X' is recognized by the ADAM HVR via binding with an associated protein 'Y'. (C) ADAM cleaves substrate 'X' in *trans* via binding with an associated protein 'Y'.

canonical ADAMs (Supplementary Figure 1). Comparison of the recently solved ADAM10 D/C-domain partial structure ($ADAM10_{D+C}$) (Janes *et al*, 2005) and that of VAP1 reveals that the atypical ADAM10 shares the continuous D_a/C_w structure and the C_h-domain scaffold with VAP1; however, it has an disordered D_s-domain and an alternate HVR structure and a different orientation between C_w- and C_h-domains (Figure 6). The locations of four of the five disulfide bridges within the C_h-domain are conserved between VAP1 and ADAM10 (Figure 6B and C) and thus, they enabled us to align the two sequences (Figure 6E). Based on this alignment, we completed entire alignments (Supplementary Figure 1) including 38 sequences of mammalian ADAMs and *Schizosaccharomyces pombe* Mde10 (Nakamura *et al*, 2004), presumably the founding member of the ADAM family in evolutionary terms. The $ADAM10_{D+C}$ structure lacks the eight residues (583–590 in ADAM10) that may form a flexible loop. However, VAP1 (Figure 6E) and the canonical ADAMs except for ADAM8 (Supplementary Figure 1) have extra 16 residues in this segment that, in part, forms a variable loop, flanked by the adjacent cysteinyl residues (Cys539 and Cys549 in VAP1) and protrudes from the main body of the C-domain (Figures 4A and 6B). The variable loop has highest temperature factor in the molecule and resembles to the disintegrin-loop, thus can be an additional protein-binding interface. The six VAP1 monomer molecules represent almost the same C_w/C_h domain orientation (data not shown), however that is distinct from that of ADAM10 (Figure 5A). Thus, the possibility whether different ADAMs have distinct C_w/C_h domain orientation remains to be established. Janes *et al* (2005) have shown that the three glutamate residues outside of HVR are essential for ADAM10-mediated ephrin proteolysis in *trans*, however, roles of the ADAM10 HVR has not been examined. An extensive molecular surface of the elongated arm structure (12 000 Å^2 for the VAP1 D/C-domains) might reveal additional protein–protein interaction interfaces other than the HVR. Multiple charged residues in the D-domain are essential for ADAM28 binding to $\alpha4\beta1$ (Bridges *et al*, 2003) and the RX_6DLPEF motif has been proposed for integrin $\alpha9\beta1$ binding (Eto *et al*, 2002). However, the D-domain portion of the C-shaped scaffold is away from the catalytic site; thus, those additional sites might not directly serve as target recognition interfaces for catalysis.

Uniquely among cell-surface proteins, ADAMs display both proteolytic and adhesive activities. The VAP1 structure reveals that these functions are spatially allocated to the ends of the unique C-shaped scaffold and face each other. This spatial allocation of the functional sites provide us insights

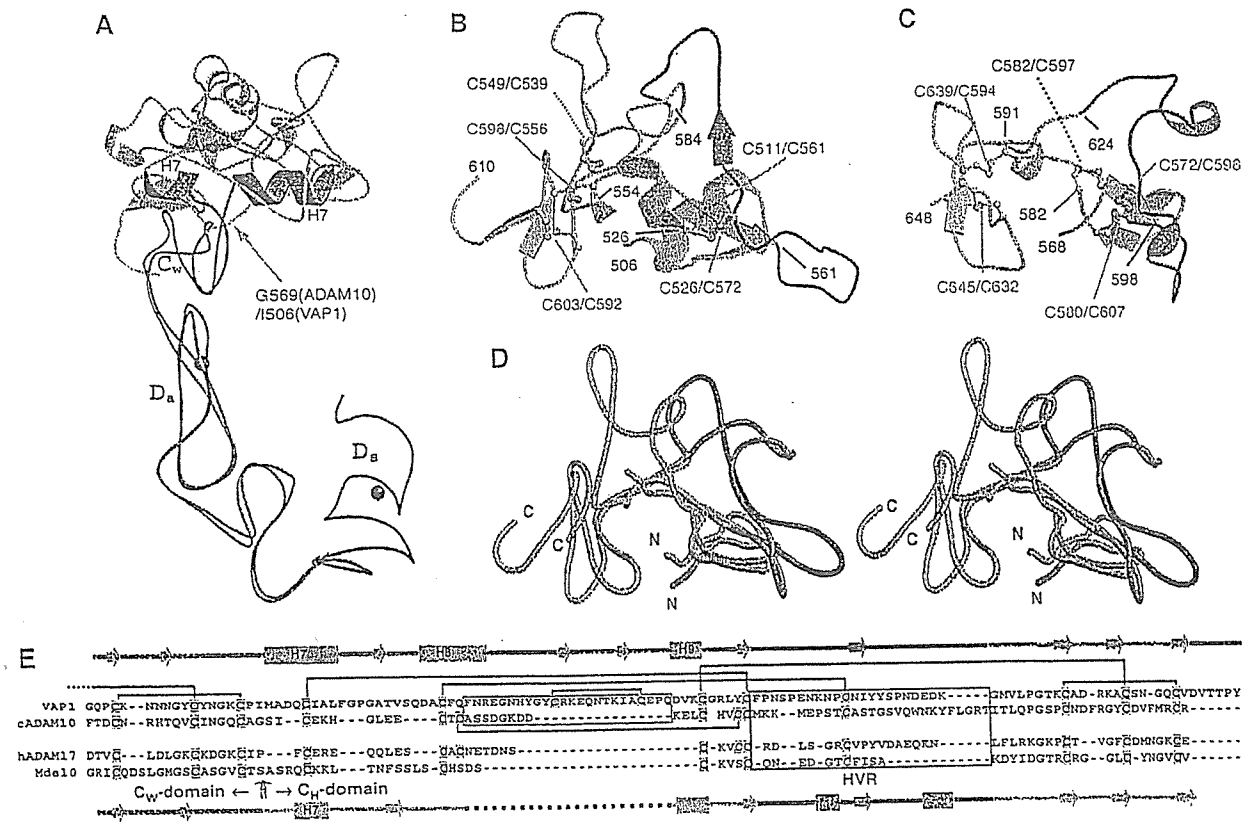

Figure 6 Comparison of the VAP1 and ADAM10 D/C domains. (A) Superimposition of the D_a-domains of ADAM10 and VAP1. The $D_s/D_a/C_w$-domains and the H7 helix of VAP1 and those of ADAM10 are shown in blue and red, respectively. The C_h-domains of VAP1 and ADAM10 are shown in cyan and pink, respectively. The arrow indicates the pivotal point between the C_w- and C_h-domains. Bound Ca^{2+} ions in VAP1 are shown as black spheres. (B) Ribbon representation of the C_h-domain of VAP1. The HVR is shown in blue. The common scaffold between the VAP1 and ADAM10 C_h-domains are shown in cyan and the segment lacking in ADAM10 is shown in light green. Disulfide bridges are indicated. (C) Ribbon representation of the C_h-domain of ADAM10. The HVR is shown in red. Disulfide brides are indicated. (D) Superimposition of the C_h-domains of VAP1 and ADAM10 *in stereo* with the colors as in (B, C). The N- and C-termini of the C_h-domains are indicated. (E) Structure-based alignments of VAP1, bovine ADAM10 (cADAM10), human ADAM17 (hADAM17) and *S. pombe* Mde10 (Mde10) C_w/C_h-domains. Secondary structures and the disulfide bridges are represented schematically. The HVR sequences and the missing segment in the ADAM10 structure are boxed in blue and green, respectively.

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into the molecular mechanism of ADAMs' target recognition, which ADAMs shed which key substrates in specific biological events. Since ADAMs are potential therapeutic targets, the distinct surface feature created by the HVR of the individual ADAMs might also provide insights into the future design of drugs with higher specificity for each member of ADAMs. We suggest that the HVR, not the disintegrin domain, should be the focus of searches for physiological targets of ADAMs.

Materials and methods

Protein preparation and crystallization

The details of the preparation, crystallization and preliminary X-ray analysis of VAP1 will be described elsewhere (T Igarashi *et al*, in preparation). VAP1 was isolated from the crude snake *Crotalus atrox* venom (Sigma-Aldrich, USA) and subjected to sitting- or hanging-drop vapor diffusion crystallization. Two distinct crystal forms ($P2_12_12_1$ and $P4_12_12$) were obtained with the reservoir solution containing 15% polyethyleneglycol 8000 and 100 mM sodium cacodylate at pH 6.5, with (orthorhombic form) or without (tetragonal form) 20 mM cobaltous chloride hexahydrate. GM6001-bound crystals were prepared by adding GM6001 (CALBIOCHEM) to the drop with the orthorhombic crystal at a final concentration qof 0.33 mM (twice the protein concentration) followed by a 12-h incubation. Crystals were flash-frozen under the nitrogen flow at 90 K.

Diffraction data collection

All the diffraction data were collected at SPring-8 beamlines using either ADSC quantum 310R CCD (for the inhibitor-bound crystal at the beamline BL41XU with $\lambda = 1$ Å), Rigaku *R*-axis V imaging plate (for orthorhombic native crystal at the beamline BL45PX with $\lambda = 1$ Å) or Jupitor CCD (for the tetragonal crystal at the beamline BL45PX with $\lambda = 0.98$ Å) detectors at 90 K. The images were reduced using HKL2000. Both orthorhombic and tetragonal native data sets were collected to 2.5-Å resolution and inhibitor-bound crystal data sets were collected to 3.0 Å resolution (Table I).

Structural analysis

All structures were solved by the molecular replacement method by MOLREP in the CCP4 suite (CCP4, 1994) by using acutolysin-C (1QUA) (Zhu *et al*, 1999) as a starting model. Initially, the MR solution obtained from the orthorhombic crystal data set, assumed two M-domains in the asymmetric units. After manual rebuilding by TURBO-FRODO, the model was subjected to tortional molecular dynamic refinements with restrained NCS averaging of the M-domains using CNS (Brunger *et al*, 1998) and iterative refinements and manual rebuilding of the model improved the electron-density map and enabled us to extend the model. First, we found the electron densities associated with the pieces of helical segments of the molecules and modelled them as poly-alanine chains. After cycles of refinements, we assigned those segments as the parts of helices H7 and H8, where the secondary structures are predicted to be helices, judging from the electron densities associated with the side chains. At this stage, four tyrosine residues, Tyr575 and Try576 within the central β strands of the HVRs were clearly defined, and we noticed that there was another NCS-axis between the C-domains. After iterative rounds of refinements with restrained NCS averaging of the C-domains and manual model building, we completed modelling of the C-domains. From this stage onward, no NCS averaging was included in the refinements. Next, we modelled the D-domains with the help of automated chain tracing using the program ARP/wARP (Perrakis *et al*, 1999) and with the structural model of trimestatin (1J2L) as a guide. After completely modelling the polypeptide chains, we noticed that isolated lobes of high electron densities surrounded by oxygen atoms occurred both in the D_s- and D_a-domains. For these sites, calcium ions fit optimally to the electron density with a refined occupancy of 100% and reasonably low *B*-values, thus, we included calcium ions in the model. We also assigned a cobalt ion, which was supplemented in the crystallization buffer for the orthorhombic crystal form, located between the M- and D_s-domains in the A molecule. The part of the carbohydrate chain linked to residue Asn218 (two *N*-acetylglocosamine (NAG) moieties) was modelled. Then, water molecules were assigned. The *VAP1* cDNA encodes a protein with 610 amino-acid residues; however, the N-terminus is processed by post-translational modification (Masuda *et al*, 1998, 2000). Here, protein sequencing of the de-blocked VAP1 molecule clarified that the Glu184 side chain was modified into a pyro-form. The electron densities associated with almost the entire molecule except for the first pyroglutamic acid were defined in either monomer within the orthorhombic crystal. In the final model, 86.1% of the residues lay in the most favorable region, 13.3% in the additionally allowed region and 0.7% in the generously allowed region of the Ramachandran plot. The tetragonal crystal and inhibitor-bound crystal were solved by MR with the domains of the refined orthorhombic apo-form as a starting model. In the final model, 83.6% (80.6%) of the residues lay in the most favorable region, 15.7% (18.9%) in the additionally allowed region and 0.7% (0.5%) in the generously allowed region for tetragonal (inhibitor-bound) crystals in the Ramachandran plot. In either crystal form, the asymmetric unit contained one dimer molecule. All six monomers had almost identical structures. Refinement statistics are shown in Table I.

PDB accession codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 2ERO, 2ERP and 2ERQ for the orthorhombic native, GM6001-bound form and tetragonal-form, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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CASE REPORT

Makiko Tanaka · Yoichi Goto · Shoji Suzuki · Isao Morii
Yoritaka Otsuka · Shunichi Miyazaki · Hiroshi Nonogi

Postinfarction cardiac rupture despite immediate reperfusion therapy in a patient with severe aortic valve stenosis

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Abstract A 74-year-old woman with severe aortic valve stenosis (AS) was admitted to our hospital because of dyspnea on exertion. On day 2, she developed acute anterior wall myocardial infarction (MI) with ST elevation. Tissue plasminogen activator (tPA) was administered 10 min after the onset of chest pain, and emergency percutaneous coronary intervention was performed to induce coronary reperfusion after another 50 min. Five hours after MI onset, however, she suddenly went into electromechanical dissociation and died from cardiac rupture. This is the first case report of postinfarct cardiac rupture with severe AS occurring in spite of instituting immediate reperfusion therapy. High intraventricular pressure may be a critical risk factor for cardiac rupture in patients with AS complicated with acute MI. Further studies are required to clarify the risk and benefit of tPA administration before percutaneous coronary intervention and the necessity of the emergency correction of AS to prevent cardiac rupture.

Key words Cardiac rupture · Acute myocardial infarction · Aortic valve stenosis · Reperfusion therapy

Introduction

Cardiac rupture occurs in 1.5%–8% of patients with acute myocardial infarction (MI) and is involved in 5%–24% of in-hospital deaths due to MI.[1] The risk factors for cardiac rupture are a first transmural MI, anterior wall MI, advanced age, female gender, the absence of collaterals, a history of hypertension, and recurrent chest pain.[1–5] Here we report on a patient with severe aortic valve stenosis (AS) who developed acute MI complicated with blow-out type cardiac rupture.

M. Tanaka · Y. Goto (✉) · S. Suzuki · I. Morii · Y. Otsuka · S. Miyazaki · H. Nonogi
Division of Cardiology, Department of Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
Tel. +81-6-6833-5012; Fax +81-6-6872-7486
e-mail: ygoto@hsp.ncvc.go.jp

Case report

A 74-year-old woman with hypertension and diabetes was admitted complaining of increasing dyspnea on exertion. Her blood pressure was 116/85 mm Hg on admission and there was no jugular venous distention or peripheral edema present. However, an S4 and a grade III systolic ejection murmur at the right second rib interspace near the right border of the sternum were audible. Electrocardiography showed a normal sinus rhythm at a rate of 82 beats/min with strain T waves in leads I, aV_L, and V_{4-6} (Fig. 1a), and a chest X-ray showed prominence of the left ventricle, with a cardiothoracic ratio of 57% and mild congestion in the upper lobes. Echocardiography also revealed severe AS, left ventricular hypertrophy, and global hypokinesia, with a fractional shortening of 21%. The estimated pressure gradient across the left ventricular outflow was 177 mm Hg and the aortic valve area was 0.3 cm^2. The patient was treated with 20 mg of intravenous furosemide and soon became free from dyspnea.

On day 2, she suffered sudden chest pain while at rest. Electrocardiography and emergency echocardiography indicated anterior wall MI (Fig. 1b). Tissue plasminogen activator (tPA; monteplase, 1 600 000 units) was administered 10 min after the onset of chest pain, intravenous nitroglycerin and heparin were given, and emergency coronary angiography was started. It was subsequently determined that the proximal left anterior descending coronary artery was occluded. Percutaneous coronary intervention (PCI) was thus performed and a metallic stent (Bx Velocity Stent with Hepacoat, 3.0 × 23 mm, Cordis, Miami, FL, USA) was inserted after predilation was carried out using a same-size balloon catheter (Maverick^2 Monorail Balloon Catheter, Boston Scientific, Natick, MA, USA). Coronary flow to the left anterior descending artery was re-established 1 h after the onset of chest pain (Fig. 2), although a distal embolic

occlusion was found at the distal end of the left anterior descending artery at the end of the PCI.

The patient's systolic blood pressure was kept strictly below 120 mm Hg during and following the PCI via the infusion of intravenous nitroglycerin. Her total creatine kinase (CK), CK-MB, and CK-MB% 4h from the onset were 4999 U/l, 238 U/l, and 4.8%, respectively. Five hours after the onset, she suddenly lost consciousness. Electrocardiography showed electromechanical dissociation, and echocardiography showed pericardial effusion with cardiac tamponade (Fig. 3). Cardiac rupture was suggested, and she underwent emergency sternotomy and open cardiac massage, while at the same time emergency percutaneous cardiopulmonary support was initiated. Despite immediate resuscitation, the patient died.

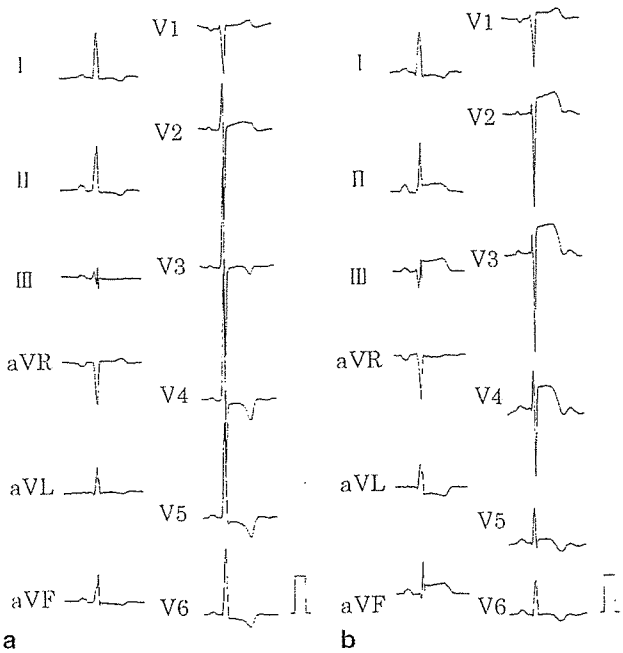

Fig. 1. Electrocardiograms of the patient. **a** The electrocardiogram on admission showed strain T waves in leads I, aV_L, and V_{4-6}. **b** ST segment elevation was observed in leads II, III, aV_F, and V_{1-5} at the onset of chest pain

Discussion

Although the patient underwent reperfusion therapy immediately after the onset of MI, she could not be rescued from catastrophic cardiac rupture, which occurred 5 h after the onset of chest pain. Blow-out rupture is characterized by the rapid development of hemodynamic collapse associated with sinus bradycardia and slow atrioventricular junctional rhythm (i.e., electromechanical dissociation), and is usually fatal. It was also difficult to keep her alive although she was subjected to full resuscitation immediately after the appearance of hemodynamic collapse.

The patient had several risk factors for postinfarct cardiac rupture such as a history of hypertension, female gender, first transmural MI, anterior wall MI, and advanced age, and these factors may have contributed to the catastrophic event. In addition to these traditional risks, she had severe AS. Several case reports have shown that postinfarct cardiac rupture occurs in patients with coexisting severe AS.[6,7] In the presence of AS, the left ventricular wall is

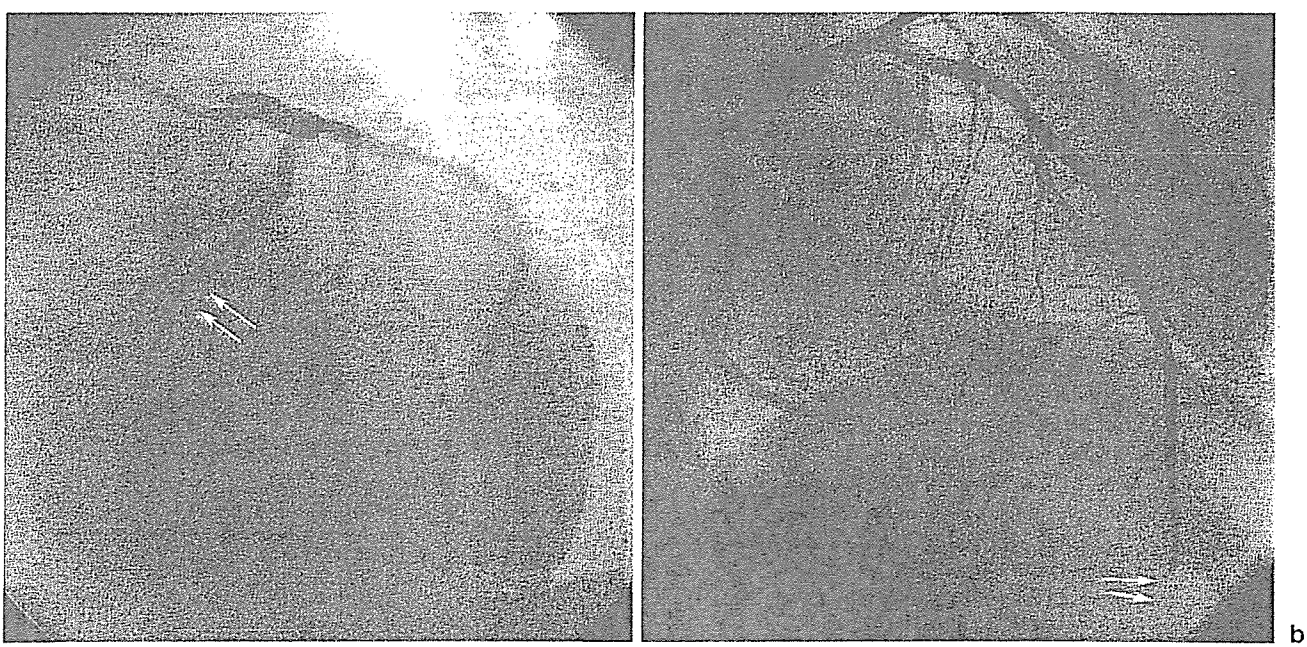

Fig. 2. **a** Coronary angiography showing an occlusion of the proximal left anterior descending coronary artery (*arrows*). **b** Coronary reperfusion was achieved in the left anterior descending artery, although a distal embolic occlusion was present (*arrows*)

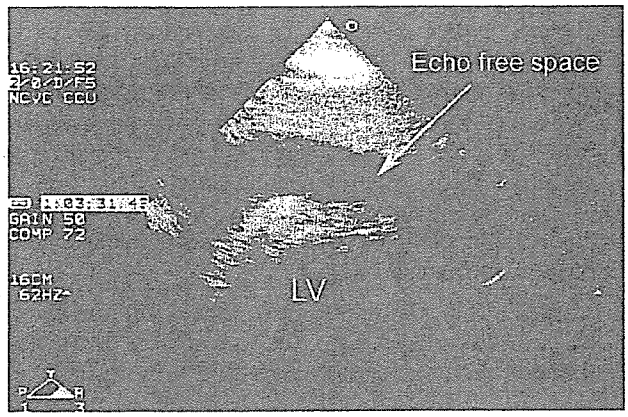

Fig. 3. Echocardiography showing pericardial effusion with findings of cardiac tamponade, indicating cardiac rupture (*arrows*). *LV*, left ventricle

subjected to an increased systolic pressure load. Furthermore, if the infarct size is small, the overall contractile strength of the left ventricle is preserved, thereby generating a high intracavitary pressure in the presence of a vulnerable infracted myocardium. High pressure in the left ventricle subsequently exhausts the infracted muscle and leads to cardiac rupture even though the peripheral blood pressure is normal. In the present case, the estimated gradient across the valve was beyond 170 mm Hg. In this situation, arterial blood pressure reduction induced by vasodilatory drugs was of no help for the compromised infarcted myocardium that had been exposed to high pressures. Cardiac rupture was inevitable even though the peripheral blood pressure had been kept strictly as low as possible during and after reperfusion therapy. Therefore, the presence of severe AS is a critical risk factor that accelerates cardiac rupture, in addition to conventional risk factors.

Generally, early recanalization reduces mortality in patients with acute MI. The PACT Trial showed that the combination therapy of short-acting reduced-dose thrombolysis and immediate planned rescue angioplasty facilitates greater LV function preservation with no significant differences in adverse events compared with primary PCI.[8] Therefore, early PCI facilitated by reduced-dose thrombolytic therapy is a beneficial and favorable strategy. On the other hand, the administration of thrombolytic drugs may increase the incidence of early cardiac rupture. In GISSI-1, the increased number of deaths during the first 6 h among patients treated with intravenous streptokinase was largely attributed to heart failure and electromechanical dissociation, and the latter was potentially a manifestation of cardiac rupture.[9] An excess of cardiac rupture events within the first 48 h was also reported in ISIS-2.[10]

Two peaks exist for the incidence of cardiac rupture after the onset of acute MI, where an early peak occurs within the first 72 h and a late peak occurs after 5–14 days.[1,11,12] Different mechanisms may be responsible for these peaks. In patients with early-phase rupture, there is hardly any thinning of the infarcted area, whereas late-phase rupture generally develops in already expanded infarcted tissue. Thrombolytic therapy may enhance the degree of early-phase rupture, although it decreases the degree of late-phase rupture and the overall death rate. The LATE study showed that, among patients treated within 12 h, the proportion of rupture deaths in the tPA group was higher than in the placebo group.[13] A large registry of these events in the United States also showed that death from cardiac rupture occurs earlier in patients treated with thrombolytic therapy, with a clustering of events within 24 h of drug administration.[14] Reperfusion may contribute to significant intramyocardial hemorrhage, which dissects through the infarcted myocardium, thus contributing to early cardiac rupture. In contrast, several studies recently found that primary direct angioplasty reduces the risk of rupture compared with thrombolysis for acute MI.[15,16] The present case had many risk factors of cardiac rupture, and the administration of tPA before PCI might have further accelerated the development of rupture no matter how early it could have been administered.

Case reports exist on patients who experienced post-MI cardiac rupture in the presence of severe AS, and who were rescued by surgical treatment.[7,17–19] However, they all had a subacute type (i.e., oozing) of cardiac rupture. No case of abrupt, catastrophic (i.e., blow-out) rupture has ever been rescued. This is the first report showing that post-MI cardiac rupture with severe AS can occur in spite of the use of immediate reperfusion therapy. Medical treatment involving immediate thrombolytic therapy followed by PCI and strict blood pressure control may have limitations in patients with severe AS. However, it remains unclear whether primary PCI alone is adequate or should be followed by the emergency correction of severe AS by aortic valve replacement or aortic valvloplasty. Further studies are thus necessary to determine an optimal treatment strategy.

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Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo

Toyotaka Yada,[1] Hiroaki Shimokawa,[3] Osamu Hiramatsu,[1] Yoshisuke Haruna,[2] Yoshitaka Morita,[2] Naoki Kashihara,[2] Yoshiro Shinozaki,[4] Hidezo Mori,[5] Masami Goto,[1] Yasuo Ogasawara,[1] and Fumihiko Kajiya[1]

[1]*Department of Medical Engineering and Systems Cardiology and* [2]*Division of Nephrology and Rheumatology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki;* [3]*Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai;* [4]*Department of Physiology, Tokai University School of Medicine, Isehara; and* [5]*Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan*

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Yada, Toyotaka, Hiroaki Shimokawa, Osamu Hiramatsu, Yoshisuke Haruna, Yoshitaka Morita, Naoki Kashihara, Yoshiro Shinozaki, Hidezo Mori, Masami Goto, Yasuo Ogasawara, and Fumihiko Kajiya. Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo. *Am J Physiol Heart Circ Physiol* 291: H1138–H1146, 2006. First published April 28, 2006; doi:10.1152/ajpheart.00187.2006.—We have recently demonstrated that endogenous H_2O_2 plays an important role in coronary autoregulation in vivo. However, the role of H_2O_2 during coronary ischemia-reperfusion (I/R) injury remains to be examined. In this study, we examined whether endogenous H_2O_2 also plays a protective role in coronary I/R injury in dogs in vivo. Canine subepicardial small coronary arteries (≥100 μm) and arterioles (<100 μm) were continuously observed by an intravital microscope during coronary I/R (90/60 min) under cyclooxygenase blockade (n = 50). Coronary vascular responses to endothelium-dependent vasodilators (ACh) were examined before and after I/R under the following seven conditions: control, nitric oxide (NO) synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA), catalase (a decomposer of H_2O_2), 8-sulfophenyltheophylline (8-SPT, an adenosine receptor blocker), L-NMMA + catalase, L-NMMA + tetraethylammonium (TEA, an inhibitor of large-conductance Ca^{2+}-sensitive potassium channels), and L-NMMA + catalase + 8-SPT. Coronary I/R significantly impaired the coronary vasodilatation to ACh in both sized arteries (both $P < 0.01$); L-NMMA reduced the small arterial vasodilatation (both $P < 0.01$), whereas it increased ($P < 0.05$) the ACh-induced coronary arteriolar vasodilatation associated with fluorescent H_2O_2 production after I/R. Catalase increased the small arterial vasodilatation ($P < 0.01$) associated with fluorescent NO production and increased endothelial NOS expression, whereas it decreased the arteriolar response after I/R ($P < 0.01$). L-NMMA + catalase, L-NMMA + TEA, or L-NMMA + catalase + 8-SPT further decreased the coronary vasodilatation in both sized arteries (both, $P < 0.01$). L-NMMA + catalase, L-NMMA + TEA, and L-NMMA + catalase + 8-SPT significantly increased myocardial infarct area compared with the other four groups (control, L-NMMA, catalase, and 8-SPT; all, $P < 0.01$). These results indicate that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role in coronary I/R injury in vivo.

endothelium-derived relaxing factor; myocardial infarction; vascular endothelial function

VASCULAR ENDOTHELIAL CELLS play an important role in maintaining vascular homeostasis by synthesizing and releasing endothelium-derived relaxing factors (EDRFs), including prostacyclin (PGI_2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) (6, 9, 26). Endothelial dysfunction is thus characterized by a reduction in the activity of PGI_2, NO, and EDHF, thereby enhancing vasoconstrictor responses mediated by endothelin, serotonin, and thrombin (26). Endothelial injury secondary to myocardial ischemia-reperfusion (I/R) decreases the production and activity of EDRFs in acute myocardial infarction (18).

Among the three different EDRFs, the roles of PGI_2 and NO have been extensively investigated (6, 9, 26). Regarding EDHF, since the first reports on its existence (6, 9), several candidates for EDHF have been proposed, including cytochrome *P*-450 metabolites (2, 4), endothelium-derived K^+ (7), and electrical communications through gap junctions between endothelial cells and vascular smooth muscle cells (29). Matoba et al. (16, 17) have previously identified that endothelium-derived H_2O_2 is a primary EDHF in mesenteric arteries of mice and humans. Morikawa et al. (21) have recently confirmed that endothelial Cu,Zn-SOD plays an important role as an EDHF synthase in mice. We have subsequently confirmed the importance of H_2O_2 in canine coronary microcirculation during coronary autoregulation with reduced coronary perfusion pressure in vivo (35).

However, it remains to be examined whether H_2O_2 also exerts cardioprotective effects during I/R in the coronary microcirculation in vivo, and if so, whether such effects of H_2O_2 compensate the impaired NO-mediated responses due to I/R injury in vivo. In this study, we tested our hypothesis that H_2O_2 plays an important cardioprotective and compensatory role during coronary I/R injury in dogs in vivo.

METHODS

This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School, and approved by an independent review committee from the same institution, and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Animal preparation. Anesthetized mongrel dogs (15–25 kg in body wt, n = 50) of either sex were ventilated with a ventilator (model VS600, IDC, Pittsburgh, PA). Aortic pressure and left ventricular (LV) pressure were continuously monitored with a catheter (SPC-784A, Millar, TX). The blood flow of the left anterior descending coronary artery (LAD) was continuously measured by a transonic flow probe (T206, Transonic Systems, Ithaca, NY).

Address for reprint requests and other correspondence: T. Yada, Dept. of Medical Engineering and Systems Cardiology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan (e-mail: yada@me.kawasaki-m.ac.jp).

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Measurements of coronary diameter by intravital microscope. We continuously monitored coronary vascular responses by an intravital microscope (VMS 1210, Nihon-Kohden, Tokyo) with a needle probe in vivo as previously described (32). We gently placed the needle probe on subepicardial microvessels. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s (32).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was measured by the nonradioactive microsphere (Sekisui Plastic, Tokyo) technique, as previously described (20). Briefly, the microsphere suspension was injected into the left atrium 85 min after the onset of coronary occlusion. Myocardial collateral flow in the apex during suturing of the collateral vessels from the left circumflex artery (LCX) was calculated according to the formula: time flow = tissue counts × (reference flow/reference counts) and was expressed in milliliters per gram per minute (20).

Detection of H_2O_2 and NO production. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF, Molecular Probes, Eugene, OR) and diaminorhodamine-4M AM (DAR, Daiichi Pure Chemicals, Tokyo) were used to detect H_2O_2 and NO production in coronary microvessels without a different NO scavenger (e.g., methylene blue), respectively, as previously described (21). Briefly, fresh and unfixed heart tissue was cut into several blocks and frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Fine Chemical, Tokyo) within a few hours. Fluorescent images of [illegible] after application of ACh by using [illegible] pus BX51, Tokyo) (21). We used different animals for the [illegible] treatment (DCF and DAR) and the 2,3,5-triphenyltetrazolium chloride (TTC) treatment.

Western blotting. Portions of myocardial samples were homogenized in lysis buffer. After centrifugation, the supernatants were used for Western immunoblotting. The proteins were transferred by semi-dry electroblotting to polyvinylidene difluoride membranes. The blots were then blocked and incubated with horseradish peroxidase-conjugated rabbit anti-endothelial NO synthase (eNOS, dimer form) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibody (Santa Cruz Biotechnology). The antibody was visualized by using an enhanced chemiluminescence method (ECL; Amersham Biosciences, Tokyo). The integrated density of the bands was quantified by using NIH Image analysis, and the protein expression level of eNOS was normalized to that of actin (24).

Experimental protocols. After the surgical procedure and instrumentation, at least 30 min was allowed for stabilization while hemodynamic variables were monitored. The following protocols were examined.

Coronary vascular responses to endothelium-dependent [ACh, 0.5 and 1.0 μg/kg intracoronary (ic)] and -independent [sodium nitroprusside (SNP), 40 and 80 μg/min ic] vasodilators were examined before ischemia (90 min)-reperfusion (60 min) (I/R). ACh and SNP were continuously and retrogradely infused into the diagonal branch of the LAD by using a syringe pump (STC 525, Terumo, Tokyo). The coronary vascular responses to ACh and SNP were examined for 2 min, and the image of maximal vasodilatation was taken at 2 min of infusion of ACh or SNP.

Coronary vasodilator responses to ACh and SNP were examined before and after coronary ischemia (90 min)-reperfusion (60 min) by proximal LAD occlusion under the following seven conditions with cyclooxygenase blockade (ibuprofen, 12.5 mg/kg iv) to evaluate the [illegible] and NO [illegible] PGI$_2$ in a different set of animals (Fig. [illegible] arginine (L-NMMA) [illegible] and 240,000 U·kg [illegible] ic [illegible] H_2O_2 into water and oxygen), *4*) adenosine receptor blockade alone [8-sulfophenyltheophylline (8-SPT), 25 μg·kg^{-1}·min^{-1} ic for 5 min], *5*) catalase plus L-NMMA, *6*) catalase plus tetraethylammonium [TEA, 10 μg·kg^{-1}·min^{-1} ic for 10 min, an inhibitor of large-

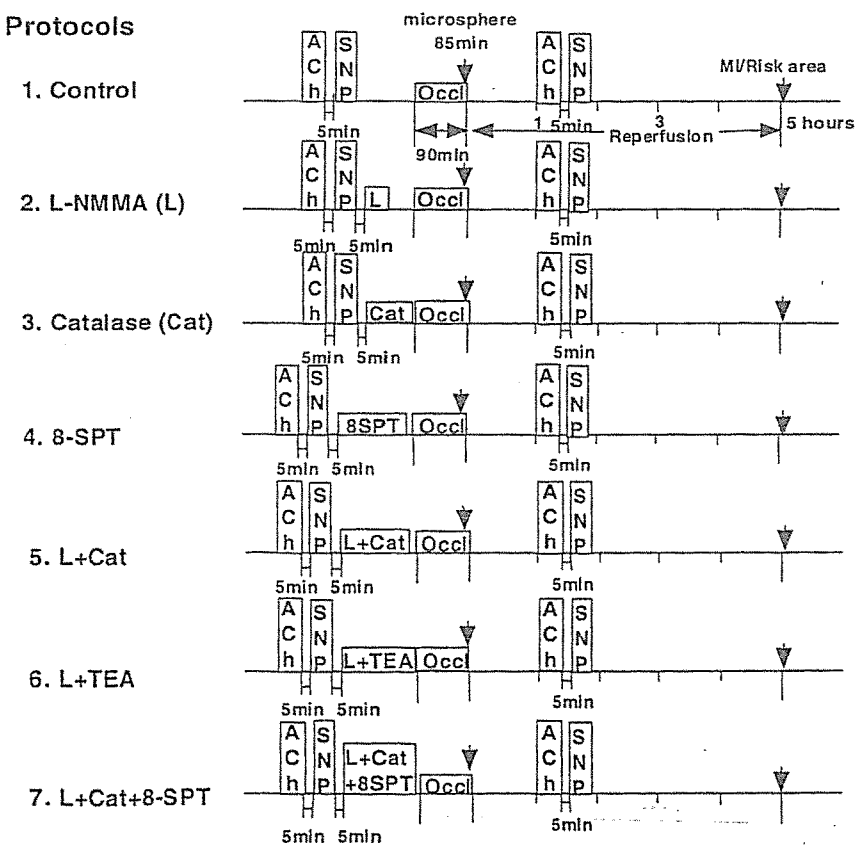

Fig. 1. Experimental protocols. TEA, tetraethylammonium; 8-SPT, 8-sulfophenyltheophylline; ACh, acetylcholine; SNP, sodium nitroprusside; Occl, coronary occlusion; Cat, catalase; L-NMMA (L), N^G-monomethyl-L-arginine; MI, myocardial infarction.

Table 1. *Hemodynamics during coronary ischemia-reperfusion injury in dogs*

		Before I/R			Ischemia (85 min)	After I/R		
	n	Baseline	ACh	SNP		Baseline	ACh	SNP
MBP, mmHg								
Control	5	92±4	91±6	92±5	93±14	92±4	91±5	92±6
L-NMMA	5	97±8	98±7	94±9	92±10	97±7	98±8	95±8
Cat	5	96±8	92±8	94±9	92±9	96±7	96±8	98±6
L-NMMA + Cat	5	94±4	93±9	97±9	95±11	95±8	98±5	94±5
L-NMMA + TEA	5	95±12	93±13	95±14	94±10	91±14	93±15	98±10
L-NMMA + Cat + 8-SPT	5	95±3	96±4	95±3	93±11	96±3	97±4	95±3
Heart rate, beats/min								
Control	5	152±5	155±3	154±3	156±7	156±5	154±5	153±5
L-NMMA	5	157±5	156±5	157±6	158±6	153±5	153±5	153±5
Cat	5	155±4	159±6	158±5	157±6	151±7	155±8	154±8
L-NMMA + Cat	5	156±12	158±13	158±13	154±5	156±13	156±14	159±13
L-NMMA + TEA	5	153±13	154±12	155±11	155±5	150±10	151±11	152±10
L-NMMA + Cat + 8-SPT	5	152±7	155±9	153±3	153±5	152±7	151±6	153±7

Results are expressed as means ± SE; *n* = no. of dogs. I/R, ischemia-reperfusion; MBP, mean blood pressure; Cat, catalase; SNP, sodium nitroprusside; TEA, tetraethylammonium; 8-SPT, sulfophenyltheophylline; L-NMMA, N^G-monomethyl-L-arginine.

conductance Ca^{2+}-sensitive potassium (K_{Ca}) channels], and *7*) catalase plus L-NMMA with 8-SPT (35). These inhibitors were given at 30 min before I/R. An interval between each treatment was 5 min. The basal coronary diameter was defined as that before administration of ACh or SNP either before or after I/R. L-NMMA, catalase, TEA, and 8-SPT were administered alone at 5 min after administration of ACh or SNP. Microspheres were administered at 85 min after the initiation of coronary occlusion. In the combined infusion (L-NMMA + catalase + 8-SPT), catalase solution was infused into the LAD at a rate of 0.5 ml/min at 5 min after infusion of L-NMMA, and then 8-SPT was added into the LAD at 15 min after the initiation of L-NMMA.

After 1 h of reperfusion, coronary vasodilator responses to ACh and SNP were examined.

After 5 h of reperfusion, we reoccluded the LAD and injected Evans blue dye into a systemic vein. Then, myocardial slices (5 μm thick) were incubated in 1% TTC (Sigma) solution to detect the infarct area (36). Different animals were used for fluorescent treatment (DCF and DAR) and TTC treatment.

Drugs. All drugs were obtained from Sigma Chemical and were diluted in a physiological saline immediately before use.

Statistical analysis. Results are expressed as means ± SE. Vascular responses (see Figs. 3*C*, 5*F*, 6*F*, 7, and 9*A*) were analyzed by one-way ANOVA followed by Scheffé's post hoc test for multiple comparisons. Difference in the effects of ACh and SNP on subepicardial coronary microvessels before and after I/R (see Figs. 3, *A* and *B*, 4, and 8, *A* and *B*), and difference between infarct size/risk area and transmural collateral flow in control and other inhibitors (see Fig. 9*B*) were examined by a multiple regression analysis by using a model in which the change in coronary diameter was set as a dependent variable (y) and vascular size as an explanatory variable (x), while the statuses of control and other inhibitors were set as dummy variables (D_1, D_2) in the following equation: $y = a_0 + a_1x + a_2D_1 + a_3D_2$, where a_0 through a_3 are partial regression coefficients (36). The criterion for statistical significance was at $P < 0.05$.

RESULTS

Hemodynamics and blood gases during I/R injury. Immediately after reperfusion, coronary blood flow was increased and some arrhythmias occurred; however, those changes returned to the control levels 1 h after reperfusion when we repeated the measurements. Thus, throughout the experiments, mean aortic pressure and heart rate at baseline were constant and comparable, and Po_2, Pco_2, and pH were maintained within the physiological ranges (pH 7.35–7.45, Po_2 > 70 mmHg, and Pco_2 25–40 mmHg,). Hemodynamic variables at baseline did not significantly change after I/R compared with those before I/R (Tables 1 and 2).

Dose responses to ACh and SNP. ACh (0.5 and 1.0 μg/kg ic) and SNP (40 and 80 μg/min ic) caused coronary vasodilatation in a dose-dependent manner at both small arteries and arterioles (Fig. 2). Then we chose the maximal dose of the vasodilators (ACh, 1.0 μg/kg ic, and SNP, 80 μg/min ic) in the following experiments.

Endothelium-dependent coronary vasodilatation before and after I/R. There was no significant difference in baseline diameter after ACh before I/R among the groups. All inhibitors did not affect resting coronary artery diameter or coronary

Table 2. *Baseline vascular diameter before I/R in response to ACh*

	Small Artery	Arteriole
Control	104–150 μm (120±7, *n* = 7)	37–96 μm (70±6, *n* = 12)
L-NMMA	106–164 μm (131±7, *n* = 8)	36–95 μm (63±5, *n* = 16)
Cat	100–147 μm (121±5, *n* = 10)	28–89 μm (61±6, *n* = 12)
8-SPT	114–162 μm (130±8, *n* = 6)	30–88 μm (60±10, *n* = 5)
L-NMMA + Cat	102–141 μm (118±5, *n* = 8)	34–95 μm (77±4, *n* = 10)
L-NMMA + TEA	105–142 μm (123±6, *n* = 5)	34–95 μm (62±9, *n* = 8)
L-NMMA + Cat + 8-SPT	110–145 μm (128±6, *n* = 5)	38–87 μm (67±7, *n* = 7)

Results are expressed as range (means ± SE); *n* = no. of blood vessels.

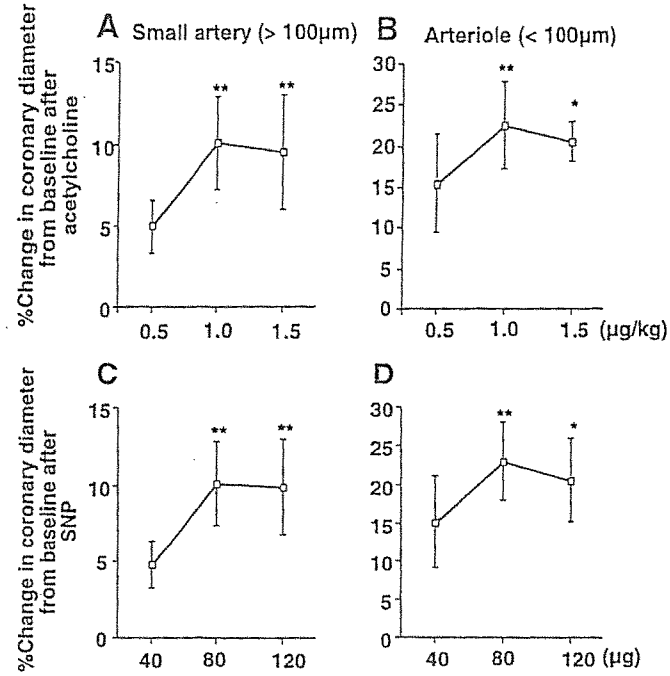

Fig. 2. Dose responses to ACh (*A* and *B*) and SNP (*C* and *D*) before ischemia-reperfusion (I/R). Number of small arteries (*A* and *C*) and arterioles (*B* and *D*) per animals used was 5/5 for each group. $*P < 0.05$, $**P < 0.01$ vs. ACh (0.5 μg/kg) and SNP (40 μg).

blood flow. Under control conditions (before I/R), intracoronary administration of ACh caused a significantly greater coronary vasodilatation in arterioles than in small arteries (Fig. 3, *A* and *B*). Coronary I/R significantly impaired the coronary vasodilatation to ACh in both sized arteries (Figs. 3*A* and 4*A*), and L-NMMA reduced the vasodilatation in small arteries (Figs. 3*A* and 4*B*) but rather increased the response in arterioles compared with control (Figs. 3*B* and 4*A*) after I/R. Catalase and 8-SPT increased the ACh-induced vasodilatation in small arteries (Figs. 3*A* and 4, *C* and *D*) but decreased the response in arterioles (Fig. 3*B*) after I/R. There was no significant difference in coronary blood flow before and after I/R among the control, the L-NMMA, and the catalase group (Fig. 3*C*). L-NMMA + catalase (Figs. 3, *A* and *B*, and 4*E*) or L-NMMA + TEA (Figs. 3, *A* and *B*, and 4*F*) decreased the vasodilatation in both sized arteries (Fig. 3, *A* and *B*) with decrement of coronary blood flow (Fig. 3*C*), and L-NMMA plus catalase with 8-SPT further decreased the vasodilatation in both sized arteries (Figs. 3, *A* and *B*, and 4*G*) compared with other groups (Fig. 3, *A–C*).

Detection of H_2O_2 and NO production. Fluorescent microscopy with DCF showed that I/R increased the vascular H_2O_2 production in control LCX (Fig. 5, *B* and *F*) compared with baseline conditions (Fig. 5, *A* and *F*) and decreased the H_2O_2 production in control LAD (Figs. 5, *C* and *F*), which was enhanced by L-NMMA (Fig. 5, *D* and *F*) and was abolished by catalase (Fig. 5, *E* and *F*) in arterioles. By contrast, the production of NO as assessed with DAR fluorescence was increased in control LCX (Fig. 6, *B* and *F*) compared with baseline LCX (Fig. 6, *A* and *F*) after I/R, decreased in control LAD (Fig. 6, *C* and *F*), inhibited by L-NMMA (Fig. 6, *D* and *F*), and was enhanced by catalase (Fig. 6, *E* and *F*) in small arteries.

Western blotting of eNOS protein expression in myocardium. In the control group, expression of eNOS protein in the ischemic LAD area was significantly decreased compared with the nonischemic LCX area (Fig. 7). In the catalase group, this decrease in the eNOS protein expression was inhibited by catalase (Fig. 7).

Endothelium-independent coronary vasodilatation. Coronary vasodilator responses to SNP were comparable under all conditions in both sized arteries (Fig. 8). Those coronary vasodilator responses were resistant to the blockade of NO synthesis with L-NMMA (Fig. 8).

Effect of H_2O_2 on I/R-induced myocardial infarct size. I/R injury caused myocardial infarction, the size of which was ~40% of the LV risk area (Fig. 9*A*). Intracoronary L-NMMA, catalase, or 8-SPT alone did not further increase the I/R-induced infarct size (Fig. 9*A*). By contrast, intracoronary L-NMMA plus catalase or TEA markedly increased the infarct size, and L-NMMA plus catalase with 8-SPT further increased

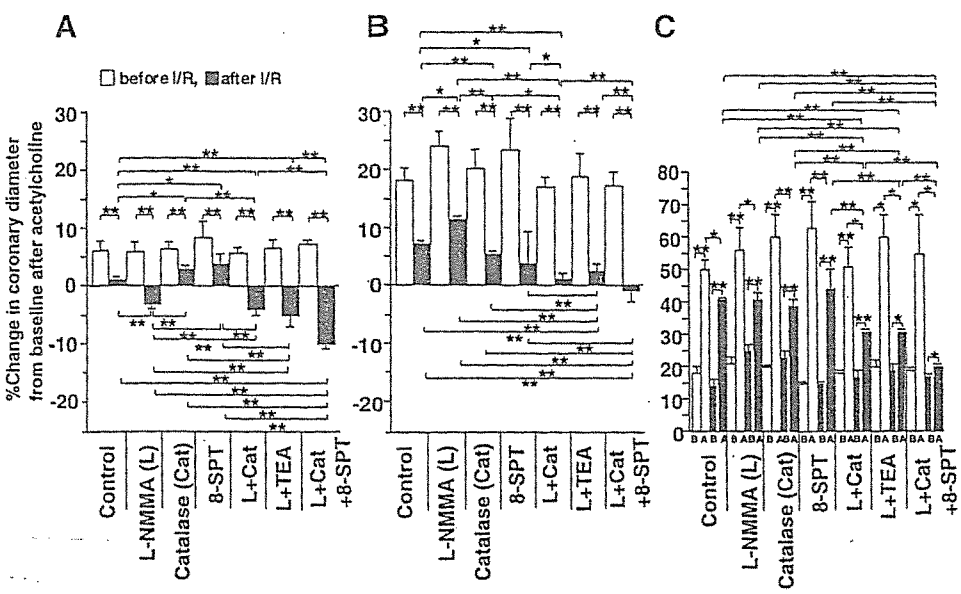

Fig. 3. Endothelium-dependent coronary vasodilatation to ACh before and after coronary I/R injury in dogs in vivo. *A*: small artery (≥100 μm). *B*: arteriole (<100 μm). *C*: coronary blood flow (CBF). No. of small arteries or arterioles per animals (*n*/*n*) used was 7/5 for control, 8/5 for L-NMMA, 10/5 for catalase, 6/5 for 8-SPT, 8/5 for L-NMMA plus catalase, 5/5 for L-NMMA plus TEA, and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries; and 12/5 for control, 16/5 for L-NMMA, 12/5 for catalase, 5/5 for 8-SPT, 10/5 for L-NMMA plus catalase, 8/5 for L-NMMA plus TEA, and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles. No. of animals during the measuring CBF used was 5 for each group. B, before ACh; A, after ACh. $*P < 0.05$, $**P < 0.01$.

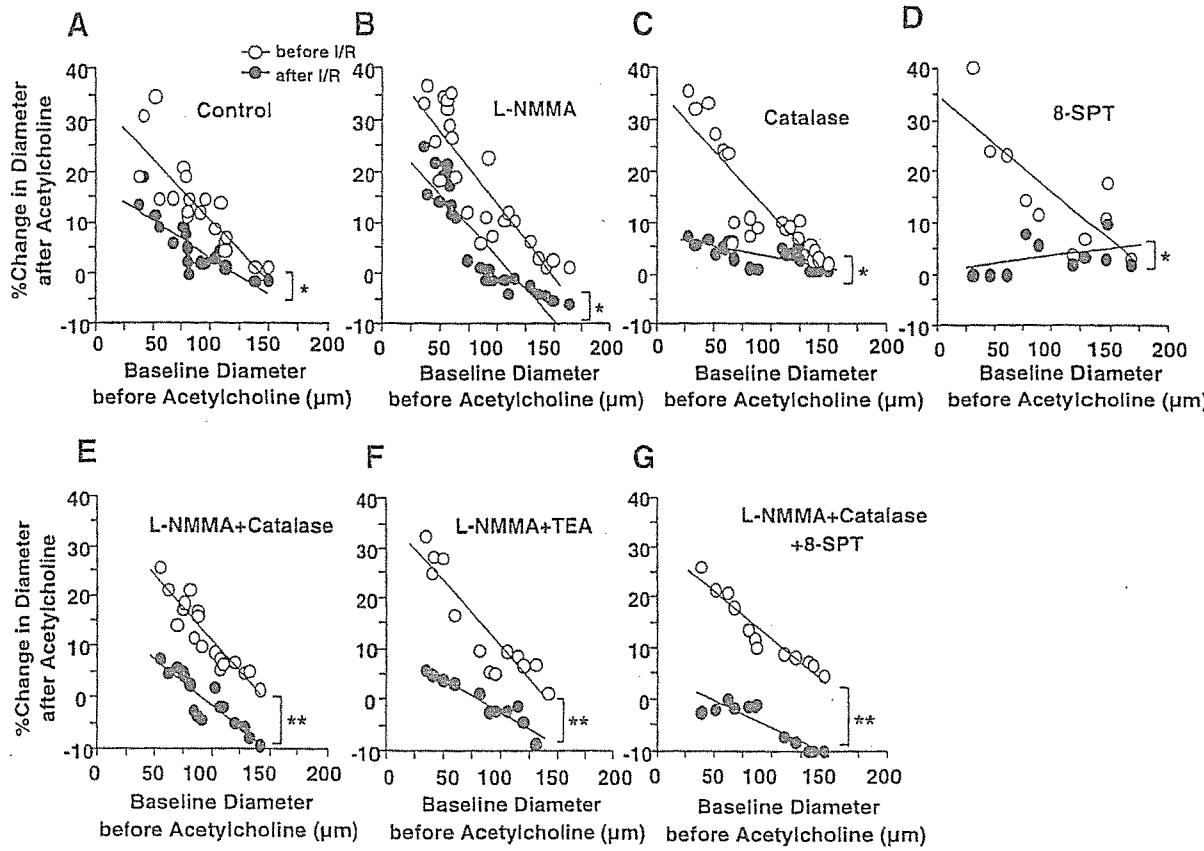

Fig. 4. Percent change in diameter after ACh before and after coronary I/R injury in dogs in vivo. No. of small arteries and arterioles per animals used was 7/5 for control (*A*), 8/5 for L-NMMA (*B*), 10/5 for catalase (*C*), 6/5 for 8-SPT (*D*), 8/5 for L-NMMA plus catalase (*E*), 5/5 for L-NMMA plus TEA (*F*), and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries (*G*); and 12/5 for control (*A*), 16/5 for L-NMMA (*B*), 12/5 for catalase (*C*), 5/5 for 8-SPT (*D*), 10/5 for L-NMMA plus catalase (*E*), 8/5 for L-NMMA plus TEA (*F*), and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles (*G*). $^*P < 0.05$, $^{**}P < 0.01$.

the infarct size (Fig. 9*A*). In the control group, there was an inverse relation between the infarct size and transmural collateral blood flow measured by microsphere technique ($r = 0.90$, $P < 0.01$). There was no significant difference in the relationship among the control, L-NMMA, and catalase treatment (Fig. 9*B*). L-NMMA plus catalase or TEA significantly shifted the regression line upward compared with the control group (both $P < 0.01$), and L-NMMA plus catalase with 8-SPT further shifted the regression line upward compared with L-NMMA plus catalase or TEA (Fig. 9*B*, both $P < 0.01$).

DISCUSSION

The major finding of the present study is that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role during coronary I/R injury as a compensatory mechanism for NO in vivo. To the best of our knowledge, this is the first report that demonstrates the important protective role of endogenous H_2O_2, in cooperation with NO, against coronary I/R injury in vivo.

Validations of experimental model and methodology. On the basis of the previous reports (22, 31), we chose the adequate dose of ACh, SNP, L-NMMA, catalase, TEA, and 8-SPT to examine the effects of endothelium-dependent and -independent coronary vasodilator responses and inhibition of NO synthesis, H_2O_2, K_{Ca} channels, and adenosine receptor, respectively. In addition, on the basis of previous studies and our own (31, 35), we choose the doses of ACh and SNP that cause maximal coronary vasodilatation in dogs in vivo. TEA at low doses is fairly specific for K_{Ca} channel, but at higher doses it may block a number of other K channels. Because several K_{Ca} channels are involved in H_2O_2-mediated responses (26), we selected the nonselective K_{Ca} inhibitor TEA to inhibit all K_{Ca} channels (15). We have previously confirmed the validity of the methods that we used in the present study (32). After 60–90 min of ischemia, ultrastructural damage of coronary endothelium was observed particularly in the subendocardium in the present study, a finding consistent with the previous study (8).

H_2O_2 during coronary I/R in vivo. It was previously reported that relaxations of isolated large canine coronary arteries to exogenous H_2O_2 were partially endothelium dependent (23). Recently, Matoba et al. (16, 17) identified that endothelium-derived H_2O_2 is an EDHF in mouse and human mesenteric microvessels. Subsequently, we (35) and others (19) have confirmed that endogenous H_2O_2 exerts important vasodilator effects in canine coronary microcirculation in vivo and in isolated human coronary microvessels, respectively. It is conceivable that H_2O_2 is produced from superoxide anions derived from several sources in endothelial cells, including eNOS, cyclooxygenase, lipoxygenase, cytochrome *P*-450 enzymes, and NAD(P)H oxidases (16). In the present study, L-NMMA or catalase alone did not com-

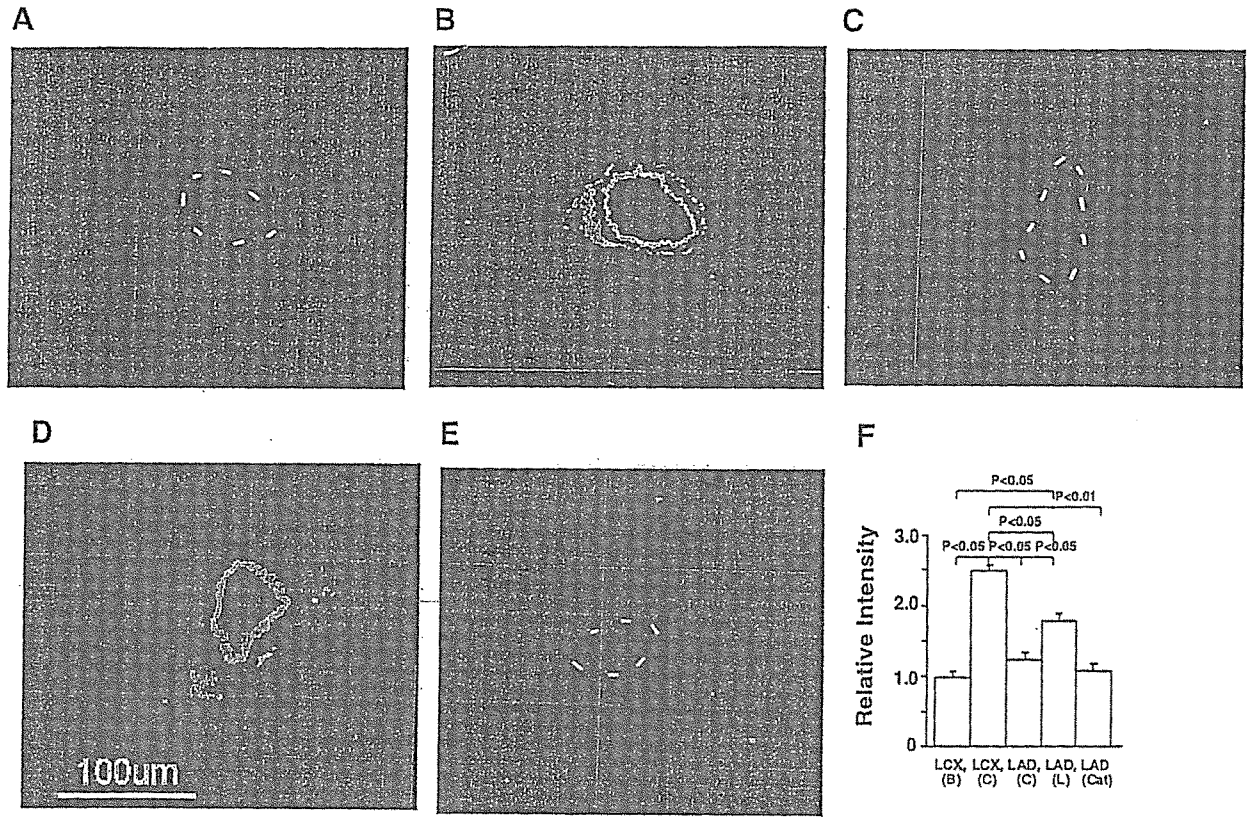

Fig. 5. Detection of H_2O_2 production. *A*: left circumflex artery (LCX; baseline without ACh). *B*: LCX (control). *C*: left anterior descending coronary artery (LAD; control). *D*: LAD (L-NMMA). *E*: LAD (catalase). *F*: fluorescent intensity (B, baseline without ACh; C, control, L, L-NMMA; Cat, catalase). No. of arterioles per animals used was 5/5 for each group. Dashed line, outline of vessels. Bar, 100 μm.

pletely abolish the ACh-induced vasodilatation in both sized arteries, whereas L-NMMA plus catalase markedly attenuated the residual vasodilatation in vivo as did TEA, indicating that H_2O_2 exerts important vasodilator effects during I/R injury in canine coronary microcirculation in vivo (Figs. 3 and 4). Furthermore, in the present study, endogenous H_2O_2-mediated coronary vasodilatation was noted to a greater extent in arterioles than in small arteries (Figs. 3 and 4), confirming the predominant role of H_2O_2 in microvessels and that of NO in relatively large arteries in vivo (25).

Compensatory vasodilator mechanism among H_2O_2, NO, and adenosine. It is well known that coronary vascular tone is regulated by the interactions among several endogenous vasodilators, including NO, H_2O_2, and adenosine (33). These vasodilators play an important role in compensatory vasodilatation of coronary microvessels during myocardial ischemia (35). In the present study (Figs. 3 and 4), endothelium-dependent arteriolar vasodilatation to ACh during coronary I/R was significantly increased by L-NMMA while small arterial vasodilatation to ACh was increased by catalase and 8-SPT, and the residual arteriolar dilation was further inhibited by both of them (L-NMMA plus catalase or TEA). Furthermore, fluorescent microscopy with DCF and DAR, respectively, showed that H_2O_2 and NO production after I/R were enhanced in small coronary arteries and arterioles by L-NMMA [fluorescent intensity (FI) 1.8] and catalase (FI 1.9) compared with those in the LAD of control group (Figs. 5 and 6, FI: DAR 1.2 and DCF 1.1). The residual small arteriolar dilatation after combined administration of L-NMMA + catalase was completely blocked by 8-SPT, an adenosine receptor blocker, indicating that adenosine also compensated for the loss of action of NO and H_2O_2. Taken together, these results indicate the compensatory vasodilator effects among NO, H_2O_2, and adenosine to maintain coronary blood flow during coronary I/R injury in vivo. H_2O_2 and NO were mutually compensatory in both small arteries and arterioles, and in the presence of their inhibitors (catalase and L-NMMA), adenosine also caused arteriolar vasodilatation, as we reported previously (35). This finding is consistent with our finding that NO, H_2O_2, and adenosine play an important compensatory role in coronary autoregulation in canine coronary microcirculation in vivo (35). It was reported that TEA inhibited adenosine-induced vasodilatation of canine subepicardial coronary arteries in vitro (3). Furthermore, H_2O_2 stimulates protein kinase C, phospholipase A_2, and arachidonic acid release and increases intracellular cAMP levels (10). These findings suggest that cAMP-mediated pathway is involved, at least in part, during coronary vasodilatation through K_{Ca} channels after I/R injury.

Role of H_2O_2 during coronary I/R. It is known that K_{Ca} channels substantially contribute to coronary vasodilatation in myocardial ischemia (22) and that H_2O_2 also activates K_{Ca} channels (11). However, it remains to be examined whether H_2O_2 contributes to coronary vasodilatation during I/R in vivo. The present results demonstrate that H_2O_2

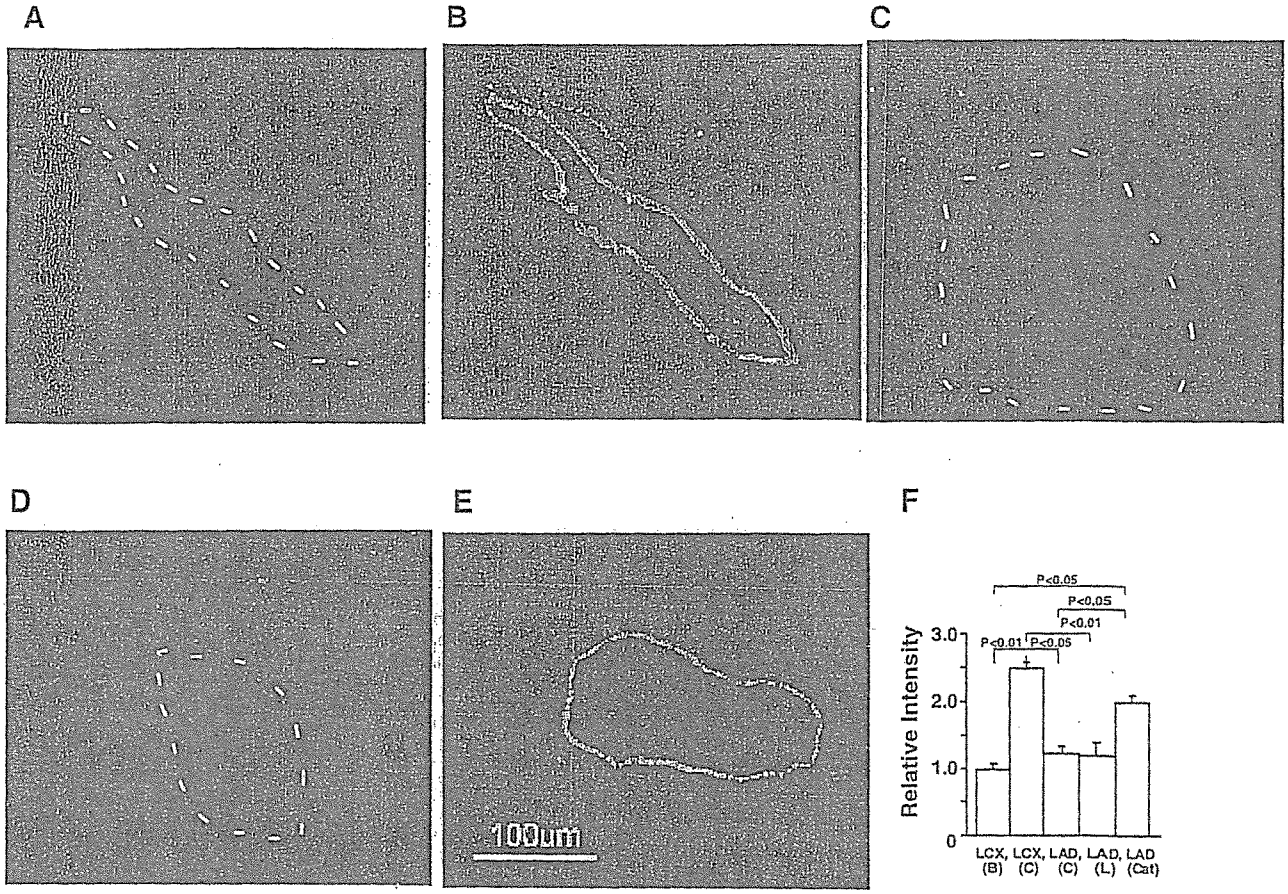

Fig. 6. Detection of nitric oxide (NO) production. *A*: LCX (baseline without ACh). *B*: LCX (control). *C*: LAD (control). *D*: LAD (L-NMMA). *E*: LAD (catalase). *F*: fluorescent intensity (B, baseline without ACh; C, control, L, L-NMMA; Cat, catalase). No. of small arteries per animals used was 5/5 for each group. Dashed line, outline of vessels.

substantially contributes to coronary vasodilatation during I/R in vivo as a compensatory mechanism for the loss of NO. Several mechanisms have been proposed for K_{Ca} channel opening during coronary I/R, including cellular acidosis

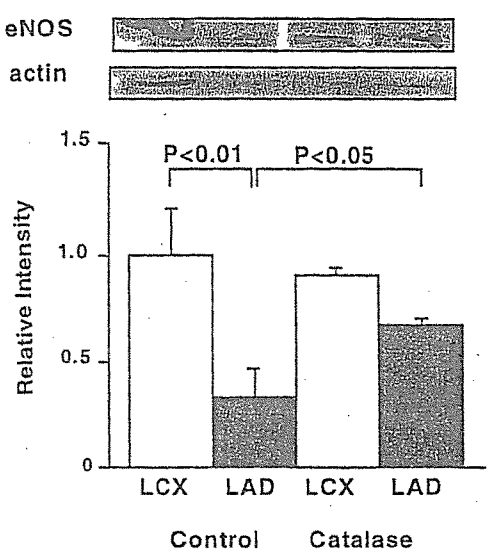

Fig. 7. Western blotting showing the effects of catalase on endothelial nitric oxide synthase (eNOS) protein expression in the myocardium of LAD and LCX. No. of animals used was 3 for each group.

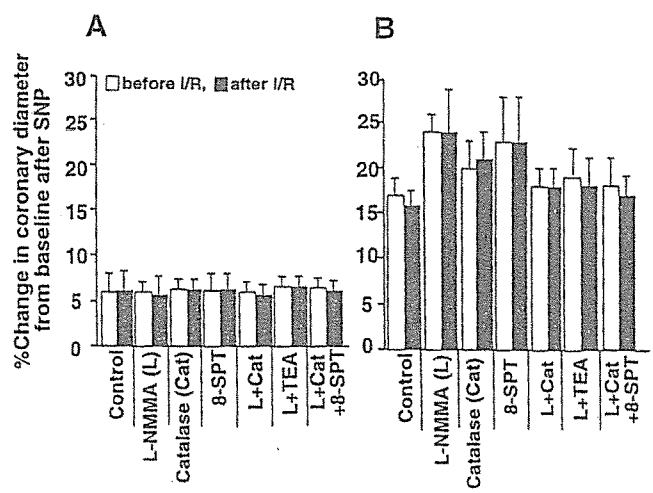

Fig. 8. Endothelium-independent coronary vasodilatation before and after coronary I/R injury in dogs in vivo. *A*: small artery (≥100 μm). *B*: arteriole (<100 μm). No. of small arteries and arterioles per animals used (*n*/*n*) was 7/5 for control, 8/5 for L-NMMA, 10/5 for catalase, 6/5 for 8-SPT, 8/5 for L-NMMA plus catalase, 5/5 for L-NMMA plus TEA, and 5/5 for L-NMMA plus catalase plus 8-SPT in small arteries; and 12/5 for control, 16/5 for L-NMMA, 12/5 for catalase, 5/5 for 8-SPT, 10/5 for L-NMMA plus catalase, 8/5 for L-NMMA plus TEA, and 7/5 for L-NMMA plus catalase plus 8-SPT in arterioles.

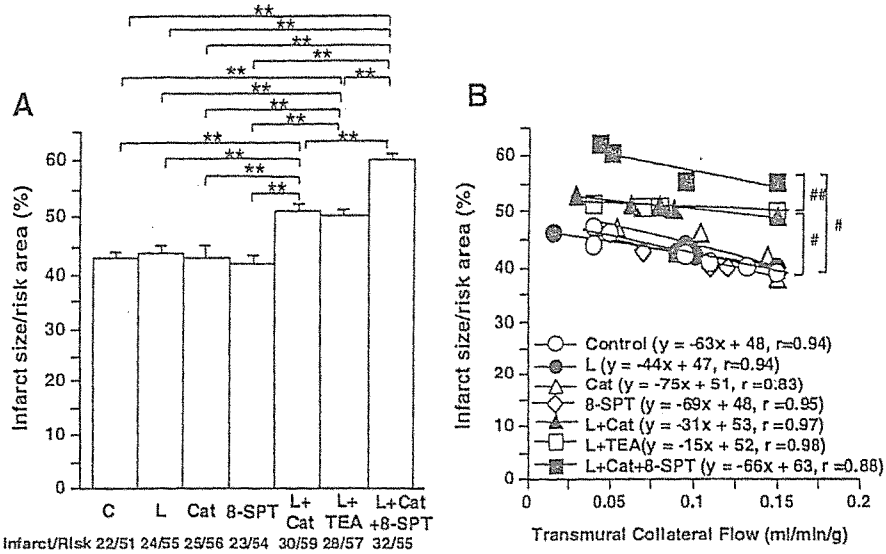

Fig. 9. Effects of H_2O_2, NO, and adenosine on I/R-induced MI in dogs in vivo. *A*: I/R-induced left ventricular infarct size in dogs in vivo. C, control. *B*: plot of infarct size expressed as a percentage of the risk area and regional collateral flow during I/R. $^{**}P < 0.01$, $\#P < 0.05$ vs. L-NMMA (L) or Cat or 8-SPT; $\#\#P < 0.01$ vs. L + TEA or L + Cat.

(27), increase in intracellular Ca^{2+} concentration after ischemia (28), and H_2O_2 production by inflammatory cells (5). Furthermore, an inhibitor of NO synthesis [N^G-nitro-L-arginine methyl ester (L-NAME)] or that of K_{Ca} channels (charybdotoxin) partly inhibits the protective effect on myocardial infarct size (22). Liu et al. (14) demonstrated that peroxynitrite inhibits K_{Ca} channel activity in human coronary arterioles during I/R. This mechanism might contribute to impaired H_2O_2-mediated dilation in I/R where NO synthase activity is increased in the presence of excess of O_2^-. In the present study, inhibition of H_2O_2 or NO alone did not significantly increase myocardial infarct size compared with control conditions (Fig. 9). These results suggest that H_2O_2 and NO exert cardioprotective effects against the development of myocardial infarction in a compensatory manner.

Recently, we have demonstrated that the expression of eNOS protein is decreased in the ischemic myocardium, which is improved by a selective Rho-kinase inhibitor, hydroxyfasudil, during coronary I/R injury in dogs in vivo (36). Furthermore, a physiological concentration (2 μmol/l) of H_2O_2 improved the recovery of both cardiac contractile function and energy metabolism after I/R in perfused rat heart (37). In the present study, the expression of eNOS protein was decreased in the ischemic myocardium, which was increased by catalase during I/R injury (Fig. 7). All these mechanisms may be involved in the beneficial effects of H_2O_2 on the I/R-induced myocardial injury. It also is conceivable that I/R reduces endothelial tetrahydrobiopterin levels in coronary vessels and impairs eNOS function (30).

Limitations of the study. Several limitations should be mentioned for the present study. First, we did not examine coronary vasodilatation in response to SOD/SOD mimetic (e.g., Tempol) or peroxynitrite inhibitor (e.g., ebselen) after I/R. However, because of the complex interactions among the oxygen species, we consider that both Tempol and ebselen also affect H_2O_2 metabolism by scavenging superoxide anions and peroxynitrite, respectively. Second, in addition to catalase, endogenous glutathione peroxidase (GSH) also plays an important role in removing H_2O_2, and NO also could be a substrate for endogenous catalase (1). However, in the present study, we used exogenous catalase to remove H_2O_2 to examine the role of the reactive oxygen species. Third, the exact source of vascular H_2O_2 production remains to be elucidated (e.g., the endothelium, smooth muscle, or cardiomyocytes). Fourth, while we were able to demonstrate the production of H_2O_2 using fluorescent microscopy with DCF, we were unable to quantitatively measure the H_2O_2 production because DCF detects H_2O_2, $ONOO^-$, and HOCl as well. Fifth, we were unable to find smaller arterioles because of the limited spatial resolution of our CCD intravital microscope. If we had an intravital camera with higher resolution, we would be able to observe coronary vasodilator responses of smaller arterioles.

Clinical implications and conclusions. During coronary I/R, microemboli of atherosclerosis debris and platelet plugs are released into the coronary microcirculation, particularly at revascularization with thrombolysis and/or percutaneous coronary intervention. Thus preexisting coronary endothelial dysfunction with various risk factors may be an important determinant for I/R injury in acute myocardial infarction. The synthesis and/or action of endothelium-derived NO are impaired under various pathological conditions, such as hypertension, hyperlipidemia, and diabetes mellitus (26, 34). In hypertension, K channel activities are increased in a compensatory manner with reduced NO activity (13). The present results suggest that NO and H_2O_2 compensate each other to cause coronary vasodilatation during I/R injury in vivo.

In conclusion, we were able to demonstrate that endogenous H_2O_2, in cooperation with NO, plays an important cardioprotective role in coronary I/R injury in vivo. The present findings may have important clinical implications because H_2O_2-mediated mechanisms substantially contribute to endothelium-dependent vasodilatation in coronary I/R in vivo.

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