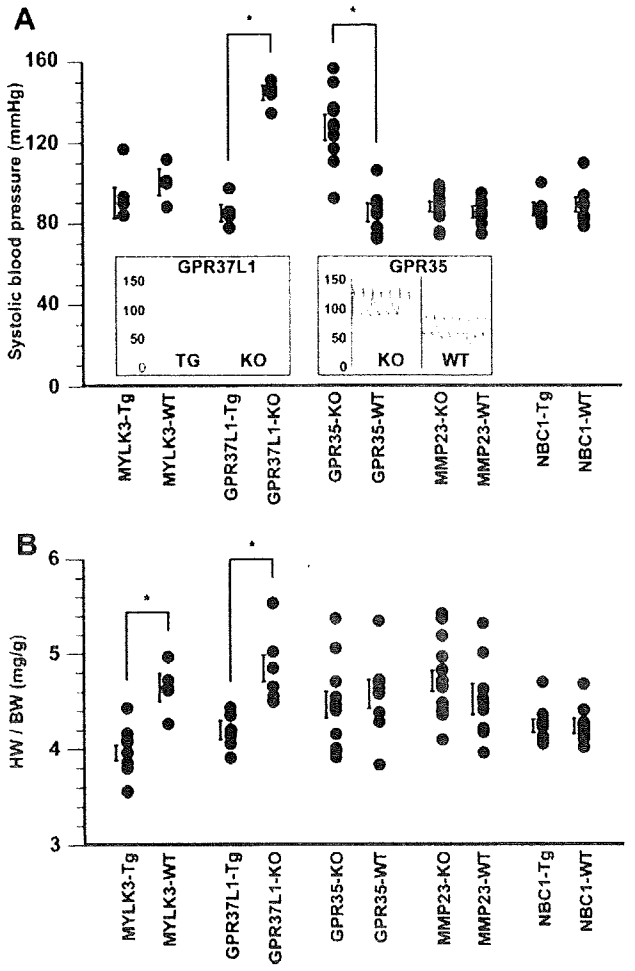

Fig. 2. *In vivo* functional analysis using gene-targeting mice of the *Mylk3*, *Gpr37l1*, *Gpr35*, *Mmp23*, and *Nbc1* genes. Blood pressure and heart weight (HW)/body weight (BW) of transgenic (Tg), knockout (KO) and their wild type (WT) littermate mice of each gene were investigated. Values are means ± SEM. $^{*}p < 0.01$. (A) Systolic blood pressure measured using Millar catheter inserted via right carotid artery. The monitoring chart shows representative data of *Gpr37l1*- and *Gpr35*-manipulated mice. (B) HW/BW ratio of each gene-targeting mouse.

the BNP mRNA level, which is the best known indicator of heart failure. The approach used in our study can help in efficient identification of the diagnostic or therapeutic targets for heart failure rather than only comparing two types of samples such as failing versus non-failing myocardium. Among the genes from these new datasets, we focused on the genes exhibiting high expression in heart tissues and finally selected four genes for performing the screening of functional analysis *in vitro*. The expression level of *MYLK3* gene was highly correlated to PAP, and this gene was detected only in the heart tissue. Recently, we reported that *MYLK3* plays a crucial role in sarcomere assembly via phosphorylation of myosin regulatory light chain 2V (MLC2v) [13]. We also showed that the knockdown of *MYLK3* by using a morpholino oligo caused immature sarcomere formation leading to ventricular dilation in zebrafish. These results indicate that *MYLK3* is strongly associated with the pathophysiology of heart failure. Chan et al. also reported that MYLK3 phosphorylates MLC2v and regulates sarcomere organization [15]. These reports affirm the reliability of our original strategy that involves the microarray analysis of failing myocardium. Among these genes, most genes including *XPR1*, *PRDX4*, and *SMOC2* have not been reported to link with cardiovascular phenotypes and were not included in many gene expression profiles published previously.

Next, we performed *in vivo* functional analysis of five selected genes, and we found that gene-targeted mouse models of *Mylk3*, *Gpr37l1*, or *Gpr35* showed the cardiovascular phenotype. As described above, *Mylk3* plays a crucial role in failing heart. In this study, we identified two GPCRs, namely, *Gpr37l1* and *Gpr35*, whose modification affects systolic blood pressure or HW/BW. To our knowledge, this is the first report about the role of these genes in cardiovascular system.

GPCRs constitute one of the largest protein families, but many GPCRs remain to be orphaned. GPR35 is now known to have some ligands such as kynurenic acid (KYNA) [16], zaprinast [17], and 5-nitro-2-(3-phenylpropylamino) benzoic acid [18]. These agonists mobilize intracellular calcium concentration. Therefore, lowering systolic blood pressure in *Gpr35*-KO mice can be induced by modulating calcium release from calcium-storing organelles. Among the three agonists, only KYNA is produced endogenously as a metabolite of tryptophan. Although GPR35 gene expression is supposed to be specific to immune cells and gastrointestinal tract, we found that GPR35 gene expression increased in failing myocardium. In an inflammatory state, interferon γ induces idolemine 2,3-dioxygenase, a rate-limiting enzyme involved in tryptophan degradation, resulting in a substantial increase in KYNA. Inflammation is thought to be involved in the pathogenesis of dilated cardiomyopathy as well as myocardial infarction. Hence there is a possibility that a KYNA-GPR35 signaling plays a role in the pathogenesis of cardiovascular diseases.

Unlike GPR35, GPR37L1 is still orphaned. However, we found that *Gpr37l1*-KO mice showed significant high blood pressure and high HW/BW as compared to Tg mice, which implies the existence of cardiovascular-related function of *Gpr37l1*. Identification of the ligand and the function of this orphan receptor are awaited.

Although no significant phenotype was observed in *Mmp23* and *Nbc1*-Tg mice, we have been investigating their cardiac function in pathological condition such as myocardial infarction or hypertension and determined their detrimental effect on heart failure (data not shown).

In the present study, we determined 12 novel heart failure-related genes by integrating an original method with parameters that indicated disease severity. Further, we assessed these possible targets of drug discovery. *MYLK3*, *GPR37L1*, and *GPR35* were the newly identified targets that play an interesting role in the cardiovascular system.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.076.

References

[1] Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. The SOLVD Investigators, N. Engl. J. Med. 325 (1991) 293–302.
[2] Effect of metoprolol CR/XL in chronic heart failure: metoprolol CR/XL randomised intervention trial in congestive heart failure (MERIT-HF), Lancet 353 (1999) 2001–2007.

[3] M. Packer, M.R. Bristow, J.N. Cohn, W.S. Colucci, M.B. Fowler, E.M. Gilbert, N.H. Shusterman, The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group, N. Engl. J. Med. 334 (1996) 1349–1355.
[4] J. Yang, C.S. Moravec, M.A. Sussman, N.R. DiPaola, D. Fu, L. Hawthorn, C.A. Mitchell, J.B. Young, G.S. Francis, P.M. McCarthy, M. Bond, Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays, Circulation 102 (2000) 3046–3052.
[5] J.D. Barrans, P.D. Allen, D. Stamatiou, V.J. Dzau, C.C. Liew, Global gene expression profiling of end-stage dilated cardiomyopathy using a human cardiovascular-based cDNA microarray, Am. J. Pathol. 160 (2002) 2035–2043.
[6] F.L. Tan, C.S. Moravec, J. Li, C. Apperson-Hansen, P.M. McCarthy, J.B. Young, M. Bond, The gene expression fingerprint of human heart failure, Proc. Natl. Acad. Sci. USA 99 (2002) 11387–11392.
[7] B.C. Blaxall, B.M. Tschannen-Moran, C.A. Milano, W.J. Koch, Differential gene expression and genomic patient stratification following left ventricular assist device support, J. Am. Coll. Cardiol. 41 (2003) 1096–1106.
[8] J.L. Hall, E.J. Birks, S. Grindle, M.E. Cullen, P.J. Barton, J.E. Rider, S. Lee, S. Harwalker, A. Mariash, N. Adhikari, N.J. Charles, L.E. Felkin, S. Polster, R.S. George, L.W. Miller, M.H. Yacoub, Molecular signature of recovery following combination left ventricular assist device (LVAD) support and pharmacologic therapy, Eur. Heart J. 28 (2007) 613–627.
[9] J. Rysa, H. Leskinen, M. Ilves, H. Ruskoaho, Distinct upregulation of extracellular matrix genes in transition from hypertrophy to hypertensive heart failure, Hypertension 45 (2005) 927–933.
[10] M.M. Kittleson, S.Q. Ye, R.A. Irizarry, K.M. Minhas, G. Edness, J.V. Conte, G. Parmigiani, L.W. Miller, Y. Chen, J.L. Hall, J.G. Garcia, J.M. Hare, Identification of a gene expression profile that differentiates between ischemic and nonischemic cardiomyopathy, Circulation 110 (2004) 3444–3451.
[11] A.S. Barth, R. Kuner, A. Buness, M. Ruschhaupt, S. Merk, L. Zwermann, S. Kaab, E. Kreuzer, G. Steinbeck, U. Mansmann, A. Poustka, M. Nabauer, H. Sultmann, Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies, J. Am. Coll. Cardiol. 48 (2006) 1610–1617.
[12] M. Asakura, M. Kitakaze, Global gene expression profiling in the failing myocardium, Circ. J. 73 (2009) 1568–1576.
[13] O. Seguchi, S. Takashima, S. Yamazaki, M. Asakura, Y. Asano, Y. Shintani, M. Wakeno, T. Minamino, H. Kondo, H. Furukawa, K. Nakamaru, A. Naito, T. Takahashi, T. Ohtsuka, K. Kawakami, T. Isomura, S. Kitamura, H. Tomoike, N. Mochizuki, M. Kitakaze, A cardiac myosin light chain kinase regulates sarcomere assembly in the vertebrate heart, J. Clin. Invest. 117 (2007) 2812–2824.
[14] M. Asakura, M. Kitakaze, S. Takashima, Y. Liao, F. Ishikura, T. Yoshinaka, H. Ohmoto, K. Node, K. Yoshino, H. Ishiguro, H. Asanuma, S. Sanada, Y. Matsumura, H. Takeda, S. Beppu, M. Tada, M. Hori, S. Higashiyama, Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy, Nat. Med. 8 (2002) 35–40.
[15] J.Y. Chan, M. Takeda, L.E. Briggs, M.L. Graham, J.T. Lu, N. Horikoshi, E.O. Weinberg, H. Aoki, N. Sato, K.R. Chien, H. Kasahara, Identification of cardiac-specific myosin light chain kinase, Circ. Res. 102 (2008) 571–580.
[16] J. Wang, N. Simonavicius, X. Wu, G. Swaminath, J. Reagan, H. Tian, L. Ling, Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35, J. Biol. Chem. 281 (2006) 22021–22028.
[17] Y. Taniguchi, H. Tonai-Kachi, K. Shinjo, Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35, FEBS Lett. 580 (2006) 5003–5008.
[18] Y. Taniguchi, H. Tonai-Kachi, K. Shinjo, 5-Nitro-2-(3-phenylpropylamino)-benzoic acid is a GPR35 agonist, Pharmacology 82 (2008) 245–249.

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Review

Mitochondrial oxidative stress and dysfunction in myocardial remodelling

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Recent experimental and clinical studies have suggested that oxidative stress is enhanced in myocardial remodelling and failure. The production of oxygen radicals is increased in the failing heart, whereas normal antioxidant enzyme activities are preserved. Mitochondrial electron transport is an enzymatic source of oxygen radical generation and can be a therapeutic target against oxidant-induced damage in the failing myocardium. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of mitochondrial DNA (mtDNA) damage as well as functional decline, further oxygen radical generation, and cellular injury. Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases. These cellular events play an important role in the development and progression of maladaptive myocardial remodelling and failure. Therefore, oxidative stress and mtDNA damage are good therapeutic targets. Overexpression of the genes for peroxiredoxin-3 (Prx-3), a mitochondrial antioxidant, or mitochondrial transcription factor A (TFAM), could ameliorate the decline in mtDNA copy number in failing hearts. Consistent with alterations in mtDNA, the decrease in mitochondrial function was also prevented. Therefore, the activation of *Prx-3* or *TFAM* gene expression could ameliorate the pathophysiological processes seen in mitochondrial dysfunction and myocardial remodelling. Inhibition of oxidative stress and mtDNA damage could be novel and effective treatment strategies for heart failure.

1. Introduction

Heart failure is a leading cause of morbidity and mortality in industrialized countries.[1] It is also a growing public health problem, mainly because of aging of the population and the increase in the prevalence of heart failure in the elderly. Previous basic, clinical, and population sciences have advanced the modern treatment of heart failure. Despite extensive studies, the fundamental mechanisms responsible for the development and progression of left ventricular (LV) failure have not yet been fully elucidated.

Reactive oxygen species (ROS) such as superoxide anions ($\cdot O_2^-$) and hydroxy radicals ($\cdot OH$) cause the oxidation of membrane phospholipids, proteins, and DNA[2] and have been implicated in a wide range of pathological conditions including ischaemia-reperfusion injury,[3] neurodegenerative diseases,[4] and aging.[5] Under physiological conditions, their toxic effects can be prevented by scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase as well as by other non-enzymatic antioxidants. However, when the production of ROS exceeds the capacity of antioxidant defenses, oxidative stress might have a harmful effect on the functional and structural integrity of biological tissue. ROS cause contractile failure and structural damage in the myocardium. The importance of oxidative stress is increasingly emerging with respect to a pathophysiological mechanism of LV remodelling responsible for heart failure progression.

Recent evidence indicates a prominent role of ROS as signalling molecules in the response to hormones, growth, and coagulation factors, cytokines, and other factors, as well as to changes in oxygen tension.[6] Many previous studies support the notion that an elevation of levels of mitochondrial ROS, during hypoxia, can control the activation of hypoxia-inducible factor (HIF)-1α. HIFs are key players in the cellular response to changes in oxygen tension. Mitochondrial ROS are intrinsically linked to HIF-1α expression during hypoxia and multiple protein kinases have the potential to be involved in the mechanism(s) by which the ROS signal is transduced to HIF-1α.[7] Recently, HIFs have also been shown to respond to non-hypoxic stimuli. However, the specific mechanisms whereby ROS accomplish this remain to be established. In addition, there were several reports that antioxidants worsened heart failure in some cases.[8]

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2. Mitochondrial oxidative stress in cardiac failure

Recent experimental and clinical studies have suggested the generation of ROS increases in heart failure.[9–12] Levels of lipid peroxides and 8-iso-prostaglandin F2α, the major biochemical markers of ROS generation, have been shown to be elevated in the plasma and pericardial fluid of patients with heart failure and also are positively correlated to its severity.[9,12] Belch *et al.*[9] reported that there was a significant negative correlation between malondialdehyde and LV ejection fraction ($r = -0.35$). Mallat *et al.*[12] assessed the functional severity of heart failure by use of the NYHA classification and the echocardiographic indices (LV diameters and the derived LV fractional shortening) and correlated these with pericardial fluid levels of 8-iso-$PGF_{2\alpha}$. Pericardial levels of 8-iso-$PGF_{2\alpha}$ were significantly increased in patients with symptomatic heart failure (NYHA II and III) compared with findings in asymptomatic patients (NYHA I) and gradually increased with the functional severity of heart failure ($P = 0.0003$). In addition, pericardial levels of 8-iso-$PGF_{2\alpha}$ were significantly correlated with LV end-diastolic and end-systolic diameters ($P = 0.008$ and 0.026, respectively).[12]

By using electron spin resonance (ESR) spectroscopy combined with the nitroxide radical, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (hydroxy-TEMPO), we definitively and directly demonstrated enhanced generation of ROS in the failing myocardium.[13] $\cdot O_2^-$ is a primary radical that could lead to the formation of other ROS, such as H_2O_2 and ·OH, in the failing myocardium. ·OH could arise from electron exchange between $\cdot O_2^-$ and H_2O_2 via the Harber–Weiss reaction. In addition, ·OH is also generated by the reduction of H_2O_2 in the presence of endogenous iron by means of the Fenton reaction. The generation of ·OH implies a pathophysiological significance of ROS in heart failure because ·OH radicals are the predominant oxidant species causing cellular injury.[14] A decreased antioxidant capacity could further aggravate the ROS accumulation in heart failure. However, the activities of SOD, catalase, and GSHPx were not decreased in the failing hearts,[15] indicating that oxidative stress in heart failure is primarily due to the enhancement of pro-oxidant generation rather than to the decline in antioxidant defenses.

The cellular sources of ROS generation within the heart include cardiac myocytes, endothelial cells, and neutrophils. Within cardiac myocytes, ROS can be produced by several mechanisms including mitochondrial electron transport, NAD(P)H oxidase, and xanthine dehydrogenase/xanthine oxidase. The heart has the highest oxygen uptake rate within the human body, consuming about 0.1 mL O_2/g per minute at basal rates.[16] To meet the demand for synthesis of ATP by oxidative metabolism, cardiac myocytes have the highest volume density of mitochondria in the entire body. Mitochondria produce ROS through a single electron transport to molecular oxygen in the respiratory chain. Under physiological conditions, small quantities of ROS are formed during mitochondrial respiration, which, however, can be detoxified by the endogenous scavenging mechanisms of myocytes.

By using ESR spectroscopy with 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap, the inhibition of electron transport at the sites of complex I and complex III in the normal submitochondrial particles resulted in a significant production of $\cdot O_2^-$.[17] Mitochondria from heart failure produced more $\cdot O_2^-$ than normal mitochondria in the presence of NADH, indicating that mitochondrial electron transport could be the predominant source of such $\cdot O_2^-$ production. Furthermore, the failing mitochondria were associated with a decrease in complex enzyme activity. Therefore, mitochondria are an important source of ROS in failing hearts, indicating a pathophysiological link between mitochondrial dysfunction and oxidative stress[18] as has been reported in other disease conditions including aging and neurodegenerative diseases.

Even though mitochondrial electron transport is considered to play an important role in ROS production in heart failure, other enzymatic sources of ROS generation such as vascular endothelial cells (via xanthine oxidase and/or NAD(P)H oxidase) and activated leukocytes (via NAD(P)H oxidase) could also contribute to oxidative stress.[19] In fact, Bauersachs *et al.*[20] have demonstrated that vascular NAD(P)H oxidase is activated in heart failure. NAD(P)H oxidase is the major source of ROS in both the endothelium and vascular smooth muscle. An increase in myocardial activity of NAD(P)H oxidase has been also observed in human heart failure.[21] Recently, by using mice lacking the cytosolic NAD(P)H oxidase component $p47^{phox}$ ($p47^{phox}-/-$ mice), Doerries *et al.*[22] demonstrated that a deficiency of the NAD(P)H oxidase and its subunit $p47^{phox}$ protected the heart from remodelling and dysfunction after myocardial infarction (MI). $p47^{phox}$ deficiency reduced LV cavity dilatation and dysfunction as well as cardiac myocyte hypertrophy, apoptosis, and interstitial fibrosis after MI, all of which contributed to improved survival. Therefore, NAD(P)H oxidase activation might also be involved in increased myocardial oxidative stress in patients with heart failure. They are able to generate ROS in response to angiotensin II, which stimulates the expression of NAD(P)H oxidase.[23,24] Plasma renin activity as well as tissue ACE activity is activated in patients with heart failure.[25,26] Therefore, an enhanced formation of angiotensin II may lead to oxidative stress via this enzyme system. Recently, Doughan *et al.*[27] provided the first evidence that angiotensin II mediate mitochondrial dysfunction in vascular endothelial cells. Angiotensin II increased mitochondrial ROS production, which was associated with decreased endothelial NO· bioavailability. Furthermore, angiotensin II-mediated mitochondrial dysfunction was dependent on activation of vascular NAD(P)H oxidases and opening of the $mitoK_{ATP}$ channels.[27]

3. Mitochondrial DNA damage, dysfunction, and oxidative stress

Mitochondria have their own genomic system, mitochondrial DNA (mtDNA), a closed-circular double-stranded DNA molecule of ~16.5 kb. MtDNA contains two promoters, the light-strand and heavy-strand promoters (LSP and HSP, respectively), from which transcripts are produced and then processed to yield the individual mRNAs encoding 13 subunits of the oxidative phosphorylation, including seven subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), one subunit (cytochrome b) of ubiquinol-cytochrome *c* oxidoreductase (complex III), three subunits

(COI, COII, and COIII) of cytochrome *c* oxidase (complex IV), and two subunits (ATPases 6 and 8) of complex V along with 22 tRNAs and two rRNA (12S and 16S) subunits.[28,29] Transcription from the LSP also produces RNA primer, which is necessary for initiating mtDNA replication. Mitochondrial function is controlled by the mtDNA as well as by factors that regulate mtDNA transcription and/or replication.[30] This raises the possibility that mtDNA damage and the impairment of mitochondrial gene transcription and/or replication are involved in heart failure. Indeed, heart failure is frequently associated with qualitative and quantitative defects in mtDNA.[31-34] Our previous studies have shown that the decline in mitochondrial function and mtDNA copy number plays a major role in the development of heart failure that occurs after MI.[17,35]

ROS can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, in addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by ROS. The mtDNA could be a major target for ROS-mediated damage for several reasons. First, mitochondria do not have a complex chromatin organization consisting of histone proteins, which may serve as a protective barrier against ROS. Second, mtDNA has a limited repair activity against DNA damage. Third, a large part of $\cdot O_2^-$, which is formed inside the mitochondria, cannot pass through the membranes and, hence, ROS damage may be contained largely within the mitochondria. In fact, mtDNA accumulates significantly higher levels of the DNA oxidation product, 8-hydroxydeoxyguanosine, than nuclear DNA.[36] As opposed to nuclear-encoded genes, mitochondrial-encoded gene expression is largely regulated by the copy number of mtDNA.[37] Therefore, mitochondrial injury is reflected by mtDNA damage as well as by a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis, and mitochondrial function.[38,39] We demonstrated that the increased generation of ROS was associated with mitochondrial damage and a dysfunction in the failing hearts, which were characterized by an increased lipid peroxidation in the mitochondria, a decreased mtDNA copy number, a decrease in the number of mtRNA transcripts, and a reduced oxidative capacity due to low complex enzyme activities.[35] Importantly, the enzymatic activities of complexes I, III, and IV all decreased in the failing hearts, whereas there was no decrease in the enzymatic activity of complex II and citrate synthase, both of which were exclusively encoded by nuclear DNA. Chronic increases in ROS production are associated with mitochondrial damage and dysfunction, which thus can lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury (*Figure 1*). MtDNA defects may thus play an important role in the development and progression of myocardial remodelling and failure.

A number of pathogenic mtDNA base substitution mutations, such as misssense mutations and mtDNA rearrangement mutations (deletions and insertions), have been identified in patients with mitochondrial diseases.[34] An accumulation of the deleted forms of mtDNA in the myocardium frequently results in cardiac hypertrophy, conduction block, or heart failure.[40] Furthermore, there is now a consensus view that mutations in mtDNA and abnormalities in mitochondrial function are associated with common forms of cardiac diseases such as ischaemic heart disease[41] and dilated cardiomyopathy.[42] In these conditions, however, the strict causal relationships between abnormalities in mtDNA and cardiac dysfunction have yet to be fully elucidated.[43] Even though the mechanisms by which mtDNA damage arises in these conditions have not been clarified, ROS have been proposed to be the primary contributing factor. We have provided direct evidence that the abnormalities in mtDNA replication/transcription as well as repair occur not only in a limited small subset of mitochondrial diseases but also in a more common form of heart failure phenotype such as that seen occurring after MI, in cases of cardiomyopathy, or in the diabetic heart.[44-46]

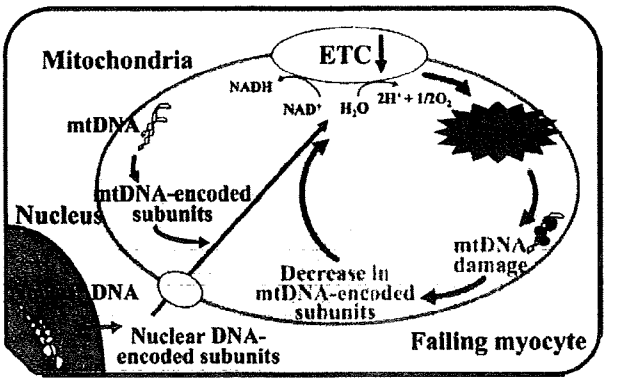

Figure 1 Schematic representation of an intimate link between reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage, and respiratory chain dysfunction in the mitochondria. Mitochondrial ROS generation may lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury. ATP, adenosine triphosphate; ETC, electron transport chain.

4. Role of mitochondrial oxidative stress in myocardial remodelling

Oxidative stress has direct effects on cellular structure and function and may activate integral signalling molecules in myocardial remodelling and failure (*Figure 2*). ROS result in a phenotype characterized by hypertrophy and apoptosis in isolated cardiac myocytes.[47]

Another potential target of ROS is matrix metalloproteinases (MMPs), a family of proteolytic enzymes. MMPs play a pivotal role in normal tissue remodelling processes, such as cell migration, invasion, proliferation, and apoptosis. They regulate many developmental processes, including branching morphogenesis, angiogenesis, wound healing, and extracellular matrix degradation. This proteolytic system is constitutively expressed in a large number of cell and tissue types and degrades a wide spectrum of extracellular matrix proteins.[48] ROS have also been shown to activate MMP in cardiac fibroblasts.[49] Myocardial MMP activity is increased in the failing hearts.[47,50] Further, an MMP inhibitor has been shown to limit early LV dilatation in a murine model of MI.[51] We have shown significant improvement in survival after MI in MMP-2 knockout mice, which was mainly attributable to the inhibition of early cardiac rupture and the development of subsequent LV dysfunction.[52] Because MMP can be activated by ROS,[53] one proposed mechanism of LV remodelling is the activation of MMPs secondary to increased ROS production. Sustained MMP activation might influence the structural properties of the myocardium by providing an abnormal extracellular environment with which the

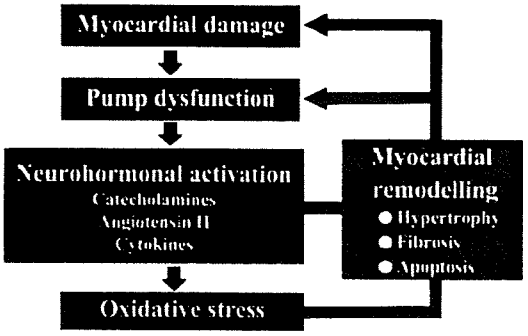

Figure 2 Role of neurohormonal activation and oxidative stress in the onset and progression of myocardial remodelling and failure.

myocytes interact. We have demonstrated that an ·OH scavenger, dimethylthiourea, inhibits the activation of MMP-2 in association with the development of LV remodelling and failure.[54] These data raise the interesting possibility that enhanced oxidative stress after MI can be a stimulus for myocardial MMP activation, which might play an important role in the development of heart failure.

5. Role of oxidative stress and skeletal muscle dysfunction in heart failure

The limitation of exercise capacity is a major symptom in patients with heart failure[55] and is independent of the degree of their cardiac dysfunction.[56] Increased oxidative stress has been shown to be related to the limitation of exercise capacity in patients with heart failure.[57] We have demonstrated that ROS are increased in the skeletal muscle of mice with heart failure after MI and that they originate from $\cdot O_2^-$ produced by mitochondria.[58] Recently, Kinugawa *et al.*[59] clarified the relationship between $\cdot O_2^-$ and the limitation of exercise capacity by using heterozygous manganese superoxide anion dismutase (SOD2) gene-knockout mice, in which SOD2, a family of enzymes that catalyse the dismutation of $\cdot O_2^-$, is reduced by 30–80%, increasing $\cdot O_2^-$ production in the mitochondria, associated with altered mitochondrial function. The whole-body oxygen consumption (VO_2) and carbon dioxide production (VCO_2) at rest were increased in $SOD2^{-/-}$. The work (vertical distance run × body weight) to exhaustion was decreased in $SOD2^{-/-}$. When the maximum VO_2 and VCO_2 were corrected to per work unit, they were increased in $SOD2^{+/-}$. Tempol, a SOD mimetic, normalized basal VO_2 and VCO_2 and improved the work to exhaustion and corrected VO_2 and VCO_2 in $SOD2^{+/-}$. There was a decrease in SOD2 protein levels and a concomitant increase in lucigenin-detectable $\cdot O_2^-$ production in skeletal muscle from $SOD2^{+/-}$. Therefore, exercise capacity was reduced in conditions in which $\cdot O_2^-$ was increased, and this was associated with a greater increase in whole-body oxygen consumption.

Uncoupling proteins (UCPs) are inner mitochondrial membrane proton transporters and decrease the proton electrochemical gradient across the inner mitochondrial membrane. Decreases in the electrochemical gradient reduce the energy force for adenosine triphosphate (ATP) biosynthesis during respiration requiring oxygen and stimulate heat production. Myocardial expression of UCP-2 was significantly increased and levels of the high-energy phosphate creatine phosphate (CrP) were decreased in heart failure.[60,61] In the failing heart, oxygen consumption is disproportionately high despite a decrease in CrP or the ratio of CrP to ATP, indicating a reduction in myocardial energy efficiency.[62] In addition, Echtay *et al.*[63] have shown that ROS inside mitochondria activate the expression of UCPs. These results suggested that ROS could cause the alteration of energy efficiency through the expression of UCP-2, which might have an important role in regulating cardiac as well as skeletal muscle function during the process of heart failure.

6. Amelioration of mitochondrial dysfunction and myocardial remodelling

6.1 GSHPx

The first line of defense mechanism against ROS-mediated cardiac injury comprises several antioxidant enzymes including SOD, catalase, and GSHPx. Among these antioxidants, GSHPx is an important enzyme that performs several vital functions. GSHPx is a key antioxidant that catalyses the reduction of H_2O_2 and hydroperoxides. ·OH arises from electron exchange between $\cdot O_2^-$ and H_2O_2 via the Harber–Weiss reaction. In addition, ·OH is also generated by the reduction of H_2O_2 in the presence of endogenous iron by means of the Fenton reaction. GSHPx scavenges H_2O_2, which results in the prevention of the formation of other more toxic radicals such as ·OH.[14] GSHPx possesses a higher affinity for H_2O_2 than catalase. Further, it is present in relatively high amounts within the heart especially in the cytosolic and mitochondrial compartments.[64] These lines of evidence imply the primary importance of GSHPx as a defense mechanism within the heart compared with catalase. Moreover, GSHPx is expected to exert greater protective effects against oxidative damage than SOD because greater dismutation of $\cdot O_2^-$ by SOD may result in an increase of H_2O_2. Therefore, compared with SOD or catalase, GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage.[65]

GSHPx gene overexpression inhibited the development of LV remodelling and failure after MI, which might contribute to the improved survival.[66] These findings not only extended the previous observation that employed antioxidants, but also revealed the major role of ROS in the pathophysiology of myocardial remodelling. These effects were associated with the attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis.[66] Similarly, overexpression of the GSHPx gene attenuated myocardial remodelling and preserved diastolic function in diabetic heart.[67] Therefore, therapies designed to interfere with oxidative stress by using GSHPx could be beneficial to prevent myocardial remodelling and failure.

6.2 Manganese superoxide dismutase

Manganese superoxide dismutase (MnSOD) is the primary mitochondrial antioxidant enzyme and is essential for maintaining normal cell development and function. Overexpression of the MnSOD gene has been shown to be beneficial in various animal models of cardiac diseases.[68,69] Recently, Shen *et al.*[70] demonstrated that protection of cardiac mitochondria by overexpression of the MnSOD gene reduced the severity of diabetic cardiomyopathy. Crossing *MnSOD*

transgenic mice with a type 1 diabetic mouse model improved respiration and normalized mass in diabetic mitochondria. MnSOD also protected the morphology of diabetic hearts and completely normalized contractility in diabetic myocytes. These results showed that elevating levels of MnSOD provided extensive protection to diabetic mitochondria and provided overall protection to the diabetic heart. Interestingly, MnSOD gene overexpression also elevated levels of myocyte catalase and mitochondrial GSH, which might also act together with MnSOD against oxidative stress.

On the contrary, Nojiri *et al.*[71] reported that heart/muscle-specific *MnSOD*-deficient mice developed progressive heart failure with specific molecular defects in mitochondrial respiration in association with excess formation of superoxide and transcriptional alterations of genes associated with heart failure. Importantly, administration of an SOD mimetic significantly ameliorated these abnormalities.

6.3 Peroxiredoxin-3

Peroxiredoxin-3 (Prx-3) is a mitochondrial antioxidant protein and member of the Prx family that can scavenge H_2O_2 in cooperation with thiol and peroxynitrite.[72] Among six known mammalian Prxs, Prx-1 to -4 require the small redox protein thioredoxin (Trx) as an electron donor to remove H_2O_2, whereas Prx-5 and -6 can use other cellular reductants, such as GSH, as their electron donor.[73] Prx-1, -2, and -6 are found in the cytoplasm and nucleus, whereas Prx-3 contains a mitochondrial localization sequence, is found exclusively in the mitochondria,[74] and uses mitochondrial Trx-2 as the electron donor for its peroxidase activity.[75] Prx-3 functions not only by removing H_2O_2 formed after the SOD-catalysed dismutation reaction but also by detoxifying peroxynitrite. Moreover, *in vivo* transfer of *Prx-3* protected neurons against cell death induced by oxidative stress.[76]

We have recently demonstrated that the overexpression of *Prx-3* protects the heart against post-MI remodelling and failure in mice. It reduces LV cavity dilatation and dysfunction as well as myocyte hypertrophy, interstitial fibrosis, and apoptosis of the non-infarcted myocardium. These beneficial effects of the Prx-3 gene overexpression were associated with the attenuation in oxidative stress, mtDNA decline, and dysfunction.[44] The specific localization of Prx-3 in the mitochondria suggests that mitochondrial oxidative stress plays an important role in the development and progression of heart failure and the antioxidant localized specifically within the mitochondria provides a primary line of defense against this disease process.

6.4 Mitochondrial transcription factor A

Mitochondrial transcription factor A (TFAM) is a nucleus-encoded protein that binds upstream of the LSP and HSP of mtDNA and promotes transcription of mtDNA. TFAM not only regulates mtDNA transcription and replication,[77] but also maintains mtDNA copy number. In fact, *Tfam* knockout mice, which had a 50% reduction in their transcript and protein levels, exerted a 34% reduction in the mtDNA copy number, 22% reduction in the mitochondrial transcript levels, and partial reduction in the cytochrome *c* oxidase levels in the heart.[78] Moreover, cardiac-specific disruption in Tfam in mice exhibited dilated cardiomyopathy in association with a reduced amount of mtDNA and mitochondrial transcripts.[79] The transfection of antisense plasmids in culture, designed to reduce the expression of *TFAM*, effectively decreased the levels of mtDNA encoded transcripts.[31] On the contrary, the forced overexpression of *TFAM* could produce the opposite effect.[80] These lines of evidence have established a critical role for TFAM in regulation of mtDNA copy number and mitochondrial function as well as maintenance of the physiological function of the heart. In addition, a reduction in *TFAM* expression has been demonstrated in several forms of cardiac failure.[32,35,46,81]

By using transgenic mice that overexpress human TFAM, we examined whether TFAM could protect the heart from mtDNA deficiencies and attenuate LV remodelling and failure after MI.[82] *TFAM* overexpression could ameliorate the decline in mtDNA copy number and preserve it at a normal level in post-MI hearts. *TFAM* overexpression might increase the steady-state levels of mtDNA by directly stabilizing mtDNA. Consistent with alterations in mtDNA, the decrease in oxidative capacities seen in MI was also prevented. Moreover, *TFAM* significantly attenuated cardiac chamber dilatation and dysfunction as well as histopathological changes such as myocyte hypertrophy, interstitial fibrosis, and apoptosis seen in heart failure. Therefore, TFAM was considered to play an important role in myocardial protection against remodelling and failure (*Figure 3*).

Several factors may be attributable to the protective effects conferred by *TFAM* overexpression against myocardial remodelling and failure. First, *TFAM* overexpression prevented the decrease in mtDNA copy number and normalized mitochondrial electron transport function, which may contribute to the decrease in oxidative stress. The decreased oxidative stress could contribute to the amelioration of cardiac hypertrophy, apoptosis, and interstitial fibrosis.[82] A recent study by Ekstrand *et al.*[83] demonstrated that the overexpression of human *TFAM* in the mouse increased mtDNA copy number. These lines of evidence imply the primary importance of TFAM as a regulatory mechanism of

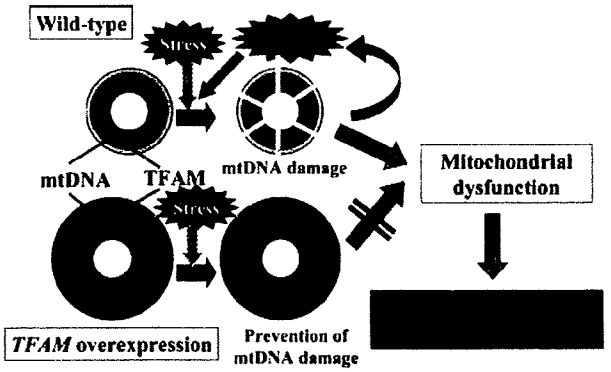

Figure 3 Proposed mechanisms through which overexpression of the mitochondrial transcription factor A (TFAM) gene prevents mitochondrial DNA (mtDNA) damage, oxidative stress, and myocardial remodelling and failure. In wild-type mice, mitochondrial transcription factor A directly interacts with mitochondrial DNA to form nucleoids. Stress such as ischaemia causes mitochondrial DNA damage, which increases the production of reactive oxygen species (ROS) and thus leads to a catastrophic cycle of mitochondrial electron transport impairment, further reactive oxygen species generation, and mitochondrial dysfunction. *TFAM* overexpression may protect mitochondrial DNA from damage by directly binding and stabilizing mitochondrial DNA and increasing the steady-state levels of mitochondrial DNA, which ameliorates mitochondrial dysfunction and thus the development and progression of heart failure.

mtDNA copy number. TFAM has been shown to directly interact with mtDNA to form nucleoids.[84,85] Therefore, increased levels of TFAM may increase the steady-state levels of mtDNA by directly binding and stabilizing mtDNA in transgenic mice. Secondly, *TFAM* overexpression may induce mitochondrial biogenesis, which, however, is thought to be unlikely because the number and size of the mitochondria assessed by electron microscopy were not altered. Nevertheless, in addition to antioxidant mechanisms, *TFAM* overexpression might have metabolic effects through increased respiratory chain activity and ATP synthesis, which is considered to exert bioenergetic improvements and be cardioprotective against heart failure.

The results obtained from human *TFAM* transgenic mice differ from those from the inducible, cardiac-specific overexpression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)) transgene in adult mice, which leads to a modest increase in mitochondrial number and development of reversible cardiomyopathy.[86] PGC-1α is the transcriptional coactivator and acts upstream of TFAM and also has the capacity to increase mtDNA levels as well as mitochondrial mass in cultured cells and in transgenic mice.[87,88] The reason for the discrepant results between *PGC-1α* and *TFAM* transgene overexpression remains unsolved, which, however, may be related to the complex regulatory mechanisms of mitochondrial biogenesis and function by PGC-1α and its downstream factors, including nuclear respiratory factors 1 and 2 and TFAM.[89,90]

7. Future perspectives

mtDNA decline, mitochondrial defects, and mitochondrial oxidative stress are now well recognized in a variety of diseases such as neurodegenerative diseases, diabetes mellitus, cancer, and even aging. Therefore, with further knowledge on the mechanisms of TFAM for maintenance of mtDNA copy number and mitochondrial function, it may eventually be possible to develop novel strategies based on the manipulation of TFAM for the treatment of diseases such as heart failure.

The sirtuin family has emerged as therapeutic targets to treat age-related diseases including cardiovascular diseases, type 2 diabetes, and neurodegenerative diseases.[91–93] There are seven human sirtuins (SIRT1–7) that display diversity in cellular localization and function. SIRT1 has been implicated as a key mediator of the pathways downstream of resveratrol and calorie restriction, a dietary regimen that is known to delay the onset and reduce the incidence of age-related diseases. Calorie restriction increases muscle mitochondrial biogenesis through SIRT1 and activation of PGC-1.[94] Recently, small-molecule activators of SIRT1 have been shown to improve glucose homeostasis, increase insulin sensitivity in liver, skeletal muscle, and fat, and increase mitochondrial function in type 2 diabetic mice.[95] The interesting possibility that these interventions can prevent or ameliorate the onset and progression of various age-related diseases including heart failure awaits further experimentation. However, Alcendor *et al.*[92] demonstrated that a high level (12.5-fold) of the Sirt1 gene increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy, whereas low (2.5-fold) to moderate (7.5-fold) overexpression of the Sirt1 gene in transgenic mouse hearts attenuated age-dependent cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers. Moderate overexpression of the Sirt1 gene protected the heart from oxidative stress induced by paraquat, with increased expression of antioxidants, such as catalase, through forkhead box O (Fox O)-dependent mechanisms.[92]

8. Summary

To improve the prognosis of patients with heart failure, we need to develop therapeutic strategies based on a novel insight into the pathophysiology of myocardial remodelling and failure. The approach of regulating mitochondrial oxidative stress and mtDNA damage may contribute to the establishment of effective treatment strategies for patients with heart failure. Oxidative stress is involved not only in heart failure, but also in various cardiovascular diseases including atherosclerosis, hypertension, and in the aging process. Therefore, therapeutic strategies to modulate this maladaptive response should become a target for future extensive investigation and could have a broad application.

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References

1. Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. *J Am Coll Cardiol* 1993;**22**:6A-13A.
2. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985;**312**:159-163.
3. Chen H, Hu CJ, He YY, Yang DI, Xu J, Hsu CY. Reduction and restoration of mitochondrial dna content after focal cerebral ischemia/reperfusion. *Stroke* 2001;**32**:2382-2387.
4. Mizuno Y, Yoshino H, Ikebe S, Hattori N, Kobayashi T, Shimoda-Matsubayashi S *et al.* Mitochondrial dysfunction in Parkinson's disease. *Ann Neurol* 1998;**44**:S99-S109.
5. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE *et al.* Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 2004;**429**:417-423.
6. Finkel T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol* 2003; **15**:247-254.
7. Sanjuan-Pla A, Cervera AM, Apostolova N, Garcia-Bou R, Victor VM, Murphy MP *et al.* A targeted antioxidant reveals the importance of mitochondrial reactive oxygen species in the hypoxic signaling of HIF-1alpha. *FEBS Lett* 2005;**579**:2669-2674.
8. Nightingale AK, Crilley JG, Pegge NC, Boehm EA, Mumford C, Taylor DJ *et al.* Chronic oral ascorbic acid therapy worsens skeletal muscle metabolism in patients with chronic heart failure. *Eur J Heart Fail* 2007;9: 287-291.
9. Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. *Br Heart J* 1991;**65**:245-248.
10. Hill MF, Singal PK. Antioxidant and oxidative stress changes during heart failure subsequent to MI in rats. *Am J Pathol* 1996;**148**:291-300.
11. Hill MF, Singal PK. Right and left myocardial antioxidant responses during heart failure subsequent to MI. *Circulation* 1997;**96**:2414-2420.
12. Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, Tedgui A. Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for in vivo oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* 1998;**97**:1536-1539.
13. Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K *et al.* Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ Res* 2000;**86**:152-157.

14. Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK *et al.* Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial 'stunning' is a manifestation of reperfusion injury. *Circ Res* 1989;**65**: 607-622.
15. Tsutsui H, Ide T, Hayashidani S, Suematsu N, Utsumi H, Nakamura R *et al.* Greater susceptibility of failing cardiac myocytes to oxygen free radical-mediated injury. *Cardiovasc Res* 2001;**49**:103-109.
16. Antoni H. Function of the heart. In: Schmidt RF, Thews G (eds). *Human Physiology.* Berlin, Heidelberg, New York: Springer-Verlag; 1991. p358-396.
17. Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N *et al.* Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res* 1999;**85**:357-363.
18. Sawyer DB, Colucci WS. Mitochondrial oxidative stress in heart failure: 'oxygen wastage' revisited. *Circ Res* 2000;**86**:119-120.
19. Munzel T, Harrison DG. Increased superoxide in heart failure: a biochemical baroreflex gone awry. *Circulation* 1999;**100**:216-218.
20. Bauersachs J, Bouloumie A, Fraccarollo D, Hu K, Busse R, Ertl G. Endothelial dysfunction in chronic MI despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production. *Circulation* 1999;**100**: 292-298.
21. Heymes C, Bendall JK, Ratajczak P, Cave AC, Samuel JL, Hasenfuss G *et al.* Increased myocardial NADPH oxidase activity in human heart failure. *J Am Coll Cardiol* 2003;**41**:2164-2171.
22. Doerries C, Grote K, Hilfiker-Kleiner D, Luchtefeld M, Schaefer A, Holland SM *et al.* Critical role of the NAD(P)H oxidase subunit p47phox for left ventricular remodeling/dysfunction and survival after MI. *Circ Res* 2007;**100**:894-903.
23. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994;**74**:1141-1148.
24. Li JM, Shah AM. Mechanism of endothelial cell NADPH oxidase activation by angiotensin II. Role of the p47phox subunit. *J Biol Chem* 2003;**278**: 12094-12100.
25. Francis GS, Benedict C, Johnstone DE, Kirlin PC, Nicklas J, Liang CS *et al.* Comparison of neuroendocrine activation in patients with left ventricular dysfunction with and without congestive heart failure. A substudy of the Studies of Left Ventricular Dysfunction (SOLVD). *Circulation* 1990;**82**: 1724-1729.
26. Zisman LS, Asano K, Dutcher DL, Ferdensi A, Robertson AD, Jenkin M *et al.* Differential regulation of cardiac angiotensin converting enzyme binding sites and AT1 receptor density in the failing human heart. *Circulation* 1998;**98**:1735-1741.
27. Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res* 2008; **102**:488-496.
28. Attardi G, Schatz G. Biogenesis of mitochondria. *Annu Rev Cell Biol* 1988; 4:289-333.
29. Shadel GS, Clayton DA. Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem* 1997;**66**:409-435.
30. Clayton DA. Replication and transcription of vertebrate mitochondrial DNA. *Annu Rev Cell Biol* 1991;**7**:453-478.
31. Kajander OA, Karhunen PJ, Jacobs HT. The relationship between somatic mtDNA rearrangements, human heart disease and aging. *Hum Mol Genet* 2002;**11**:317-324.
32. Lebrecht D, Setzer B, Ketelsen UP, Haberstroh J, Walker UA. Time-dependent and tissue-specific accumulation of mtDNA and respiratory chain defects in chronic doxorubicin cardiomyopathy. *Circulation* 2003; **108**:2423-2429.
33. Naya FJ, Black BL, Wu H, Bassel-Duby R, Richardson JA, Hill JA *et al.* Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor. *Nat Med* 2002;**8**:1303-1309.
34. Wallace DC. Mitochondrial diseases in man and mouse. *Science* 1999;**283**: 1482-1488.
35. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K *et al.* Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* 2001;**88**: 529-535.
36. Giulivi C, Boveris A, Cadenas E. Hydroxyl radical generation during mitochondrial electron transfer and the formation of 8-hydroxydesoxyguanosine in mitochondrial DNA. *Arch Biochem Biophys* 1995;**316**:909-916.
37. Williams RS. Mitochondrial gene expression in mammalian striated muscle. Evidence that variation in gene dosage is the major regulatory event. *J Biol Chem* 1986;**261**:12390-12394.
38. Ballinger SW, Patterson C, Yan CN, Doan R, Burow DL, Young CG *et al.* Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res* 2000;**86**:960-966.
39. Williams RS. Canaries in the coal mine: mitochondrial DNA and vascular injury from reactive oxygen species. *Circ Res* 2000;**86**:915-916.
40. Anan R, Nakagawa M, Miyata M, Higuchi I, Nakao S, Suehara M *et al.* Cardiac involvement in mitochondrial diseases. A study on 17 patients with documented mitochondrial DNA defects. *Circulation* 1995;**91**: 955-961.
41. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. *Mutat Res* 1992;**275**:169-180.
42. Arbustini E, Diegoli M, Fasani R, Grasso M, Morbini P, Banchieri N *et al.* Mitochondrial DNA mutations and mitochondrial abnormalities in dilated cardiomyopathy. *Am J Pathol* 1998;**153**:1501-1510.
43. Clayton DA, Williams RS, Liang I. Meeting highlights. *Circulation* 1995;**92**: 2022-2023.
44. Matsushima S, Ide T, Yamato M, Matsusaka H, Hattori F, Ikeuchi M *et al.* Overexpression of mitochondrial peroxiredoxin-3 prevents left ventricular remodeling and failure after myocardial infarction in mice. *Circulation* 2006;**113**:1779-1786.
45. Li YY, Chen D, Watkins SC, Feldman AM. Mitochondrial abnormalities in tumor necrosis factor-alpha-induced heart failure are associated with impaired DNA repair activity. *Circulation* 2001;**104**:2492-2497.
46. Kanazawa A, Nishio Y, Kashiwagi A, Inagaki H, Kikkawa R, Horiike K. Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart. *Am J Physiol Endocrinol Metab* 2002; **282**:E778-E785.
47. Spinale FG, Coker ML, Thomas CV, Walker JD, Mukherjee R, Hebbar L. Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: relation to ventricular and myocyte function. *Circ Res* 1998;**82**:482-495.
48. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* 2000;**14**:2123-2133.
49. Siwik DA, Tzortzis JD, Pimental DR, Chang DL, Pagano PJ, Singh K *et al.* Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes in vitro. *Circ Res* 1999;**85**:147-153.
50. Creemers EE, Cleutjens JP, Smits JF, Daemen MJ. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 2001;**89**:201-210.
51. Rohde LE, Ducharme A, Arroyo LH, Aikawa M, Sukhova GH, Lopez-Anaya A *et al.* Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. *Circulation* 1999;**99**:3063-3070.
52. Hayashidani S, Tsutsui H, Ikeuchi M, Shiomi T, Matsusaka H, Kubota T *et al.* Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction. *Am J Physiol Heart Circ Physiol* 2003;**285**:H1229-H1235.
53. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996;**98**:2572-2579.
54. Kinugawa S, Tsutsui H, Hayashidani S, Ide T, Suematsu N, Satoh S *et al.* Treatment with dimethylthiourea prevents left ventricular remodeling and failure after experimental myocardial infarction in mice: role of oxidative stress. *Circ Res* 2000;**87**:392-398.
55. Sullivan MJ, Green HJ, Cobb FR. Skeletal muscle biochemistry and histology in ambulatory patients with long-term heart failure. *Circulation* 1990;**81**:518-527.
56. Wilson JR. Exercise intolerance in heart failure. Importance of skeletal muscle. *Circulation* 1995;**91**:559-561.
57. Nishiyama Y, Ikeda H, Haramaki N, Yoshida N, Imaizumi T. Oxidative stress is related to exercise intolerance in patients with heart failure. *Am Heart J* 1998;**135**:115-120.
58. Tsutsui H, Ide T, Hayashidani S, Suematsu N, Shiomi T, Wen J *et al.* Enhanced generation of reactive oxygen species in the limb skeletal muscles from a murine infarct model of heart failure. *Circulation* 2001; **104**:134 136.
59. Kinugawa S, Wang Z, Kaminski PM, Wolin MS, Edwards JG, Kaley G *et al.* Limited exercise capacity in heterozygous manganese superoxide

dismutase gene-knockout mice: roles of superoxide anion and nitric oxide. *Circulation* 2005;**111**:1480-1486.
60. Noma T, Nishiyama A, Mizushige K, Murakami K, Tsuji T, Kohno M *et al.* Possible role of uncoupling protein in regulation of myocardial energy metabolism in aortic regurgitation model rats. *FASEB J* 2001;15: 1206-1208.
61. Murakami K, Mizushige K, Noma T, Tsuji T, Kimura S, Kohno M. Perindopril effect on uncoupling protein and energy metabolism in failing rat hearts. *Hypertension* 2002;**40**:251-255.
62. Takaoka H, Takeuchi M, Odake M, Hata K, Hayashi Y, Mori M *et al.* Depressed contractile state and increased myocardial consumption for non-mechanical work in patients with heart failure due to old myocardial infarction. *Cardiovasc Res* 1994;**28**:1251-1257.
63. Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA *et al.* Superoxide activates mitochondrial uncoupling proteins. *Nature* 2002;**415**:96-99.
64. Le CT, Hollaar L, van der Valk EJ, van der Laarse A. Buthionine sulfoximine reduces the protective capacity of myocytes to withstand peroxide-derived free radical attack. *J Mol Cell Cardiol* 1993;**25**:519-528.
65. Toussaint O, Houbion A, Remacle J. Relationship between the critical level of oxidative stresses and the glutathione peroxidase activity. *Toxicology* 1993;**81**:89-101.
66. Shiomi T, Tsutsui H, Matsusaka H, Murakami K, Hayashidani S, Ikeuchi M *et al.* Overexpression of glutathione peroxidase prevents left ventricular remodeling and failure after myocardial infarction in mice. *Circulation* 2004;**109**:544-549.
67. Matsushima S, Kinugawa S, Ide T, Matsusaka H, Inoue N, Ohta Y *et al.* Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart. *Am J Physiol Heart Circ Physiol* 2006;**291**:H2237-H2245.
68. Yen HC, Oberley TD, Vichitbandha S, Ho YS, St Clair DK. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J Clin Invest* 1996;**98**: 1253-1260.
69. Chen Z, Siu B, Ho YS, Vincent R, Chua CC, Hamdy RC *et al.* Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J Mol Cell Cardiol* 1998;**30**:2281-2289.
70. Shen X, Zheng S, Metreveli NS, Epstein PN. Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. *Diabetes* 2006;**55**:798-805.
71. Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S *et al.* Oxidative stress causes heart failure with impaired mitochondrial respiration. *J Biol Chem* 2006;**281**:33789-33801.
72. Bryk R, Griffin P, Nathan C. Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 2000;**407**:211-215.
73. Fisher AB, Dodia C, Manevich Y, Chen JW, Feinstein SI. Phospholipid hydroperoxides are substrates for non-selenium glutathione peroxidase. *J Biol Chem* 1999;**274**:21326-21334.
74. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J Biol Chem* 1998;**273**: 6297-6302.
75. Watabe S, Hiroi T, Yamamoto Y, Fujioka Y, Hasegawa H, Yago N *et al.* SP-22 is a thioredoxin-dependent peroxide reductase in mitochondria. *Eur J Biochem* 1997;**249**:52-60.
76. Hattori F, Murayama N, Noshita T, Oikawa S. Mitochondrial peroxiredoxin-3 protects hippocampal neurons from excitotoxic injury in vivo. *J Neurochem* 2003;**86**:860-868.
77. Scarpulla RC. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta* 2002;**1576**:1-14.
78. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M *et al.* Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* 1998;**18**:231-236.
79. Wang J, Wilhelmsson H, Graff C, Li H, Oldfors A, Rustin P *et al.* Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. *Nat Genet* 1999;**21**:133-137.
80. Montoya J, Perez-Martos A, Garstka HL, Wiesner RJ. Regulation of mitochondrial transcription by mitochondrial transcription factor A. *Mol Cell Biochem* 1997;**174**:227-230.
81. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R. Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *J Physiol* 2003;**551**:491-501.
82. Ikeuchi M, Matsusaka H, Kang D, Matsushima S, Ide T, Kubota T *et al.* Overexpression of mitochondrial transcription factor a ameliorates mitochondrial deficiencies and cardiac failure after myocardial infarction. *Circulation* 2005;**112**:683-690.
83. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K *et al.* Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet* 2004;**13**:935-944.
84. Alam TI, Kanki T, Muta T, Ukaji K, Abe Y, Nakayama H *et al.* Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res* 2003;**31**: 1640-1645.
85. Takamatsu C, Umeda S, Ohsato T, Ohno T, Abe Y, Fukuoh A *et al.* Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep* 2002;**3**:451-456.
86. Russell LK, Mansfield CM, Lehman JJ, Kovacs A, Courtois M, Saffitz JE *et al.* Cardiac-specific induction of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha promotes mitochondrial biogenesis and reversible cardiomyopathy in a developmental stage-dependent manner. *Circ Res* 2004;**94**:525-533.
87. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O *et al.* Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 2002;**418**:797-801.
88. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V *et al.* Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999;**98**:115-124.
89. Huss JM, Kelly DP. Nuclear receptor signaling and cardiac energetics. *Circ Res* 2004;**95**:568-578.
90. Ventura-Clapier R, Garnier A, Veksler V. Energy metabolism in heart failure. *J Physiol* 2004;**555**:1-13.
91. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A *et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006;**444**:337-342.
92. Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X *et al.* Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007;**100**: 1512-1521.
93. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 2004;**305**: 1010-1013.
94. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F *et al.* Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 2006;**127**: 1109-1122.
95. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ *et al.* Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 2007;**450**:712-716.

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Intravenous administration of nicorandil immediately before percutaneous coronary intervention can prevent slow coronary flow phenomenon

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Aims To determine the effect of intravenous administration of nicorandil on slow coronary flow (SCF) phenomenon in patients undergoing percutaneous coronary intervention (PCI).

Methods and results In a preliminary study, 6 mg of nicorandil showed optimal efficacy for vasodilatation without causing significant haemodynamic instability. In the main study, a total of 408 patients were randomly assigned to receive intravenous administration of 6 mg of nicorandil immediately before PCI. The number of patients in the nicorandil group was 206 [acute coronary syndrome (ACS): 47, non-ACS: 159] and that in the control group was 202 (ACS: 61, non-ACS: 141). Nicorandil significantly decreased the incidence of post-procedural SCF phenomenon in both the ACS and non-ACS groups. The rate of target vessel revascularization (TVR) was significantly lower in the nicorandil group than in the control group in ACS patients.

Conclusion Our simple procedure prevented SCF phenomenon not only in patients with ACS but also in patients with non-ACS without any adverse effect. Additionally our procedure reduced the rate of TVR in patients with ACS.

Keywords Slow coronary flow phenomenon • Nicorandil • Percutaneous coronary intervention • Acute coronary syndrome

Introduction

Slow coronary flow (SCF) phenomenon is a coronary microvascular disorder characterized by the delayed passage of contrast medium in the absence of obstructive epicardial coronary disease. Slow coronary flow phenomenon during percutaneous coronary intervention (PCI) leads to severe complications such as acute myocardial infarction (AMI), cardiogenic shock, and death.[1–5] Various factors are thought to be responsible for the development of SCF phenomenon, including intensive microvascular spasm, distal microembolization by fibrin or platelets, myocardial oedema compressing the capillary lumen, leucocyte intravascular plugging, and reperfusion injury with loss of microvascular integrity.[6,7]

Recently, several studies have demonstrated that intravenous or intracoronary administration of nicorandil, a hybrid of adenosine triphosphate-sensitive potassium (K_{ATP}) channel opener and nitrates, could reduce the incidence of this phenomenon in patients with acute coronary syndrome (ACS).[8–10] However, a standard procedure for preventing SCF phenomenon in PCI not only for ACS patients but also for non-ACS patients has not been established yet.

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Thus, we assessed the effect of intravenous administration of nicorandil on SCF phenomenon in patients undergoing emergent PCI for ACS and in patients undergoing elective PCI for non-ACS.

Methods

Patients

We performed a double-blinded, randomized prospective clinical trial at the Department of Cardiology, National Hospital Organization Okayama Medical Center between November 2004 and December 2005. Patients were divided into the following two groups: ACS group and non-ACS group. The ACS group consisted of patients with AMI and unstable angina pectoris (UAP), and the non-ACS group consisted of patients with stable angina pectoris (SAP). The diagnosis of AMI was based on episodes of chest pain persisting at least 30 min but no longer than 24 h, ST-segment elevation with at least two continuous electrocardiogram leads, and more than two-fold creatine kinase (CK) elevation above the maximum peak in the normal range. Unstable angina pectoris was defined as typical precordial chest pain at rest, angiographic evidence of a stenosis >75% according to the American Heart Association (AHA) classification[11] and no increase in serum CK activity. Stable angina pectoris was defined by symptoms of typical precordial chest pain only on effort and coronary angiographic findings of a stenosis >75% according to the AHA classification. The coronary angiographic criterion was a visual estimate by the operators. If opinions were split on whether PCI should be done, PCI was performed when quantitative coronary angiography showed a stenosis >50%. Patients with systolic blood pressure (BP) <80 mmHg, patients treated with an aspiration device because of a floating thrombus, and patients who had undergone coronary artery bypass grafting in the past or had undergone PCI during this study period were excluded. All patients gave written informed consent for participation in the study. The study protocol was approved by the hospital ethics committee.

Protocol of the preliminary study for determining the effective dose of nicorandil

In order to determine the effective dose of nicorandil, the time average of spectral peak velocity (APV) was defined using a 0.014 in. Doppler guidewire (Flowire, Cardiometrics, Inc., Mountain View, CA, USA) under intravenous administration of 1, 3, and 6 mg of nicorandil in 12 patients with 75% stenosis of the coronary artery with simultaneous recordings of heart rate (HR) and BP. Patients who underwent PCI because of SAP during the month of October in 2004 were selected for this preliminary study. These patients were not included in the main study. All 12 patients received each of the three doses. The Doppler guidewire was advanced distal to the stenotic lesion. Audio signals from the Doppler guidewire were processed with a commercially available system to determine APV. Nicorandil was injected intravenously over a 20 s period at 5 min intervals. All patients also gave written informed consent for participation in the study.

Protocol of the main study for evaluating the efficacy of intravenous administration of nicorandil in a large number of patients

A flow diagram of the progress through the phases is shown in *Figure 1*. By the concealed envelope method, 450 patients were randomized to a nicorandil or a control group. A total of 408 patients who were scheduled to undergo emergent PCI for ACS (64 patients with AMI and 44 patients with UAP) or elective PCI for non-ACS (300 patients with SAP) were assigned to receive 6 mg of nicorandil dissolved in 20 mL of 0.9% saline in the nicorandil group and 20 mL of 0.9% saline in the control group immediately before the first balloon inflation or stent implantation. The randomization schedule involved sealed, sequentially numbered envelopes that contained the treatment allocation following a computer-generated random sequence. A person not involved in the study prepared the envelopes and placed them in the catheter laboratory for the researchers. After obtaining informed consent, a PCI cardiologist asked catheter laboratory physicians who did not participate in any other part of the study protocol to open the next envelope and independently prepare and administer the drug. Double blinding was achieved by the use of identically appearing vials with colourless and clear solution in both groups. Other investigators responsible for treatment or assessment for patients were unaware of treatment allocation throughout the study. The number of patients in the nicorandil group was 206 (including 47 patients with ACS and 159 patients with non-ACS) and the number of patients in the control group was 202 (including 61 patients with ACS and 141 patients with non-ACS). After arterial access had been achieved, heparin (100 U/kg) was administered. Additional heparin was administered if the procedure lasted >60 min. Intracoronary administration of 2–5 mg of isosorbide dinitrate was performed for all patients before the initial angiograms to achieve maximal vasodilatation. Isosorbide dinitrate was the only agent except for nicorandil used before stent implantation. (No patients underwent thrombolysis or distal protection or received glycoprotein IIb/IIIa inhibitors, adenosine, or verapamil.) After the guidewire had crossed the stenotic lesion, 6 mg of nicorandil was administered by intravenous hand-injection over a 20 s period for patients in the nicorandil group 1 min before stent implantation. Implantation of stents was performed in almost all of the patients. Six patients (three in the nicorandil group and three in the control group) with non-ACS received only balloon angioplasty because the target vessels were too small for stent implantation. In these cases, nicorandil was administered 1 min before first balloon inflation. Balloon pre- or post-dilatation was undertaken at the operator's discretion. Patients were asked to start combined antiplatelet therapy with aspirin (100 mg/day) and ticlopidine (200 mg/day) >3 days before the procedure of elective PCI or from the day when emergent PCI was performed.

The primary endpoint was the incidence of post-procedural SCF phenomenon and no-reflow phenomenon immediately after stent implantation. In the six patients who received only balloon angioplasty, it was evaluated immediately after the first balloon inflation. Corrected TIMI frame count (cTFC) determined by angiography was used to assess coronary flow. Using a cineprojector equipped with a frame counter, the cTFC was measured as the number of cineframes required for the contrast medium to first reach standardized distal coronary landmarks in the culprit artery.[12] In this study, the filming speed was 15 frames/s. Slow coronary flow was defined as cTFC > 20 frames in the absence of mechanical obstruction.[13,14] Angiographical no-reflow phenomenon was defined as an acute reduction in flow (at least one grade, as defined by the TIMI trial) in the absence of mechanical obstruction.[3] The injection of contrast medium was performed by hand. Two experienced observers (H.M. and M.M.) who were blinded to random assignment assessed each angiogram for quantitative frame counts.

Secondary endpoints were the incidence of post-procedural SCF phenomenon in each of the groups (ACS and non-ACS), cTFC, serum maximum CK and CK-MB levels in AMI patients, target vessel revascularization (TVR) (re-PCI or CABG), and major

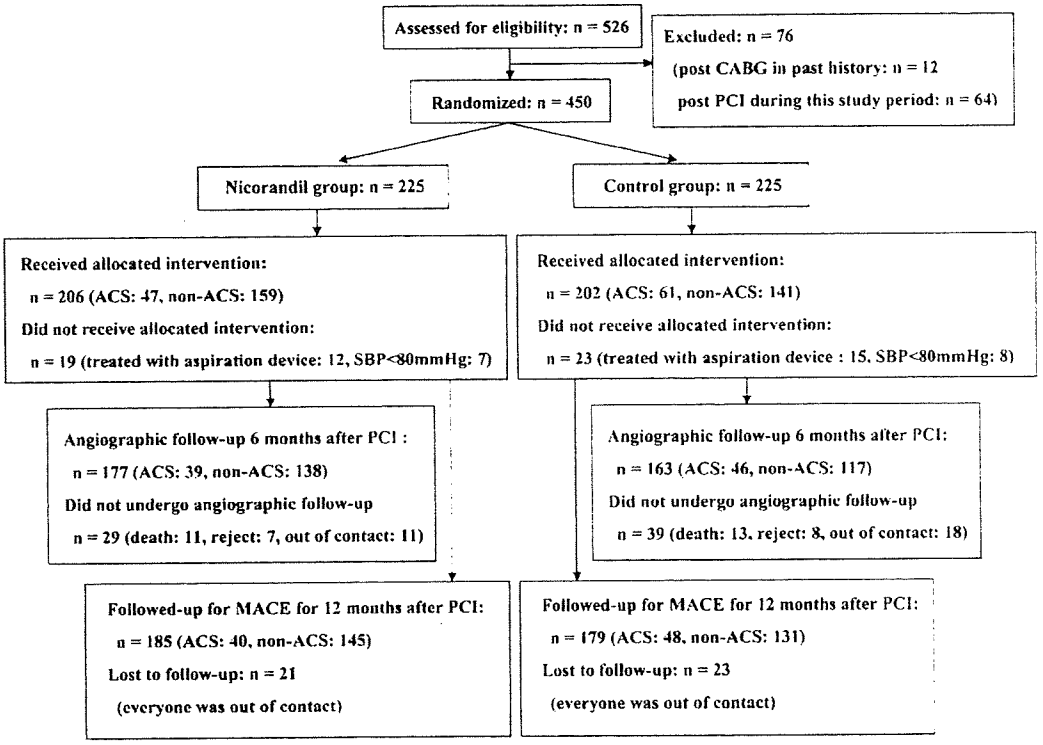

Figure 1 Flow diagram of the progress through the phases of this trial.

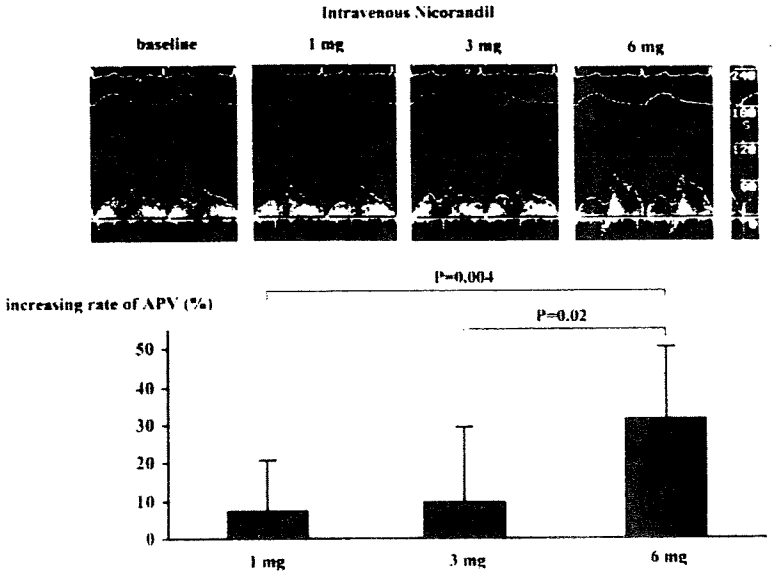

Figure 2 Top, representative flow velocity signal distal to the stenotic lesion. Bottom, dose–response effects of bolus administration of nicorandil in 12 patients with non-ACS (acute coronary syndrome).

Table 1 Baseline patient characteristics, preceding oral medications, and target lesions

	ACS		Non-ACS	
	N (n = 47)	C (n = 61)	N (n = 159)	C (n = 141)
Age (years)	69 ± 11.1	71 ± 11.5	72 ± 8.5	73 ± 9.6
Male	39 (82.9)	43 (70.5)	122 (76.7)	106 (75.2)
DM	24 (51.1)	18 (29.5)	69 (43.4))	53 (37.6)
HL	19 (40.4)	23 (37.7)	65 (40.9)	62 (44.0)
HT	32 (68.1)	46 (75.4)	112 (70.4)	108 (76.6)
Smoking	16 (34.0)	25 (41.0)	53 (33.3)	54 (38.3)
Oral medication				
ACEI	6 (12.8)	16 (26.2)	42 (26.4)	50 (35.5)
ARB	11 (23.4)	13 (21.3)	34 (21.4)	22 (15.6)
Beta-blocker	7 (14.9)	14 (23.0)	44 (27.7)	48 (34.0)
Calcium blocker	20 (42.6)	15 (24.6)	68 (42.8)	54 (38.3)
Statin	11 (23.4)	9 (14.8)	47 (29.1)	48 (34.0)
Aspirin	25 (53.2)	27 (44.3)	136 (85.5)	116 (82.3)
Glibenclamide	9 (19.1)	9 (14.8)	19 (11.9)	22 (15.6)
Culprit lesion				
RCA	18 (38.3)	27 (44.3)	60 (37.8)	45 (31.9)
LAD	20 (42.6)	23 (37.7)	61 (38.4)	59 (41.8)
LCX	9 (19.1)	11 (18.0)	35 (22.0)	35 (24.8)
LMT	0 (0.0)	0 (0.0)	3 (1.9)	2 (1.4)

Values are given as means ± SD or n (%). N, nicorandil group; C, control group; DM, diabetes mellitus; HL, hyperlipidaemia; HT, hypertension; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin 2 receptor blockers; LAD, left anterior descending coronary artery; RCA, right coronary artery; LCX, left circumflex artery; LMT, left main trunk artery.

adverse cardiac events (MACE) for 12 months after PCI. Samples for measurement of serum CK and CK-MB levels were obtained at admission and every 6 h until 48 h after PCI. Maximum CK and CK-MB levels were determined from those measurements. Revascularization was performed for symptomatic lesions with coronary angiographic findings of a stenosis >75% according to the AHA classification or non-symptomatic lesions with positive findings of stress myocardial perfusion imaging. Major adverse cardiac events included cardiovascular deaths, all deaths, or unplanned admission to hospital for management of worsening congestive heart failure (CHF). Follow-up data were obtained from hospital charts and telephone interviews with the patients, with loss of 21 patients in the nicorandil group and 23 patients in the control group during the follow-up period.

Statistical analysis

Sample size was determined assuming incidence of SCF of 15% in the control group and 6% in the nicorandil group, with 80% statistical power at a 5% level of significance. This assumption was based on results of previous studies regarding incidence of no-reflow phenomenon during PCI for ACS and non-ACS.[3,4,7] Accordingly, we calculated that the total study population should be 406.

All values are expressed as mean ± SD or frequency unless stated otherwise. Comparison between the two groups was performed by the unpaired t-test for continuous variables and by Fisher's exact test or χ^2 test for categorical variables. We estimated treatment effects by calculating relative risks for binary outcomes and differences in means for continuous data. We also calculated their corresponding 95% confidence intervals (CI). All statistical tests were two-sided, and P-values of <0.05 were considered statistically significant.

Results

Preliminary study

Intravenous administration of 6 mg of nicorandil significantly increased APV compared with the effects of 1 and 3 mg of nicorandil without causing significant haemodynamic instability (*Figure 2*). No life-threatening arrhythmia was observed during nicorandil administration.

Patients' characteristics

The total number of target lesions was 408 (47 in ACS patients and 159 in non-ACS patients in the nicorandil group, 61 in ACS patients and 141 in non-ACS patients in the control group). The baseline characteristics, preceding oral medications, and target lesions of patients in the nicorandil and control groups are shown in *Table 1*.

Haemodynamics

There were no significant differences in systolic BP, diastolic BP, and HR immediately after stent implantation between the nicorandil group and the control group (124 ± 25 vs. 126 ± 23 mmHg, $P = 0.47$; 65 ± 14 vs. 67 ± 12 mmHg, $P = 0.16$; and 73 ± 15 vs.

Table 2 The incidence of post-procedural slow coronary flow phenomenon [corrected TIMI frame count (cTFC) > 20 frames], cTFC, and serum maximum creatine kinase (CK)-MB levels in acute myocardial infarction patients

	Total				ACS				Non-ACS			
	N (n = 206)	C (n = 202)	Relative risk	P-value	N (n = 47)	C (n = 61)	Relative risk	P-value	N (n = 159)	C (n = 141)	Relative risk	P-value
SCF (%)	9 (4.4)	36 (17.8)	0.23 (0.11–0.49)	<0.0001	2 (4.3)	16 (26.2)	0.16 (0.04–0.67)	0.003	7 (4.4)	20 (14.2)	0.30 (0.12–0.72)	0.004
	Total				ACS				Non-ACS			
	N (n = 206)	C (n = 202)	Difference	P-value	N (n = 47)	C (n = 61)	Difference	P-value	N (n = 159)	C (n = 141)	Difference	P-value
cTFC	10.5 ± 5.6	12.8 ± 7.4	2.3 (1.3–3.7)	<0.0001	11.7 ± 5.8	14.9 ± 9.8	3.2 (0.44–6.2)	0.02	10.2 ± 5.5	12.1 ± 6.3	1.9 (0.83–3.4)	0.002
	AMI											
	N (n = 27)	C (n = 37)	Difference	P-value								
max CK	1767 ± 1272	2974 ± 2484	1207 (192–2223)	0.02								
max CK-MB	166 ± 122	260 ± 180	94 (14–174)	0.02								

71 ± 16 per min, $P = 0.13$, respectively). There was no significant hypotension in the nicorandil group that required administration of a pressor agent or other intensive treatment.

Incidence of post-procedural slow coronary flow phenomenon and no-reflow phenomenon

The incidence of post-procedural SCF phenomenon (cTFC > 20 frames) was significantly lower in patients in the nicorandil group than in patients in the control group (4.4 vs. 17.8%) (*Table 2*). Angiographical no-reflow phenomenon did not occur in the enrolled patients. No cases of SCF phenomenon were secondary to abrupt closure, dissection, spasm, or significant stenosis of the original target lesion.

Incidence of post-procedural slow coronary flow phenomenon in each of the groups

The incidence of post-procedural SCF phenomenon was significantly lower in both ACS and non-ACS patients in the nicorandil group than in those in the control group (4.3 vs. 26.2% and 4.4 vs. 14.2%, respectively) (*Table 2*).

Corrected TIMI frame count

The cTFC was also significantly lower in the nicorandil group than in the control group (10.5 ± 5.6 vs. 12.8 ± 7.4) (*Table 2*). The cTFC was significantly lower in both ACS and non-ACS patients in the nicorandil group than in those in the control group (11.7 ± 5.8 vs. 14.9 ± 9.8 and 10.2 ± 5.5 vs. 12.1 ± 6.3, respectively). Distribution of individual responses is shown in *Figure 3*.

Serum maximum creatine kinase and creatine kinase-MB levels in acute myocardial infarction patients

The number of patients with AMI in the nicorandil group was 27 and that in the control group was 37. The serum maximum CK and CK-MB levels were significantly lower in the nicorandil group than that in the control group (1767 ± 1272 vs. 2974 ± 2484 IU/mL and 166 ± 122 vs. 260 ± 180 IU/mL, respectively) (*Table 2*).

Target vessel revascularization

The rates of TVR are shown in *Table 3*. It was significantly lower in the nicorandil group than in the control group in ACS patients (10.3 vs. 30.4%).

Major adverse cardiac events for 12 months after percutaneous coronary intervention

Major adverse cardiac events for 12 months after PCI are shown in *Table 4*. There was no significant difference in the rate of cardiovascular deaths, all deaths, and unplanned admissions to hospital for management of worsening CHF.

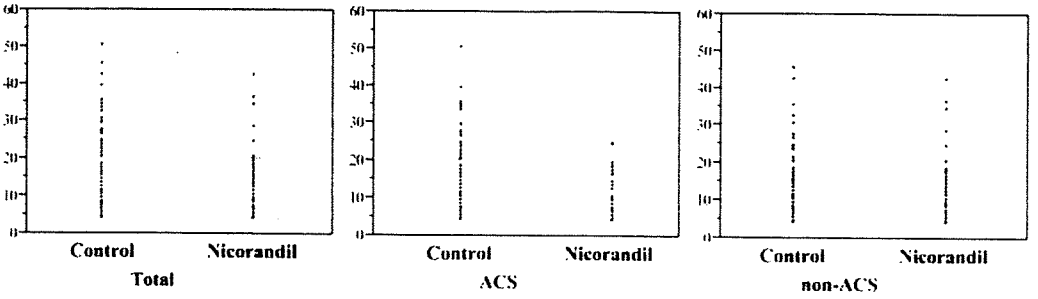

Figure 3 Distribution of individual corrected TIMI frame count.

Discussion

The major finding of this study was that intravenous administration of 6 mg of nicorandil immediately before PCI could prevent SCF phenomenon without causing any adverse effect.

It has been reported that no-reflow or SCF phenomenon is a severe complication of PCI that occurs in 11.5–37% of patients with ACS after revascularization.[3–5,8,15,16] Though SCF phenomenon sometimes occurs after revascularization in not only patients with ACS but also patients with non-ACS,[17,18] the frequency of SCF phenomenon in patients with non-ACS has not been clarified. In this study, post-procedural SCF phenomenon occurred in 14.2% of the patients with non-ACS who had not been administered intravenous nicorandil. We selected cTFC after PCI in the target artery for objective evaluation of coronary flow as a continuous quantitative variable. Corrected TIMI frame count was assessed not only as a continuous variable but also as a categorical variable with a cut-off of <20 frames. Antman *et al.*[13] reported that 28 frames is the upper 95% CI for normal flow and that 40 frames is the breakpoint between TIMI 3 and TIMI 2 flow. Because the filming speed was 30 frames/s in their study, whereas it was 15 frames/s in our study, we used 20 frames as the cut-off point between SCF and normal flow. The use of cTFC for comparison of angiographic outcomes might be superior to the use of TIMI flow grade in terms of statistical power and sensitivity.[12] According to our results, a method for preventing SCF phenomenon in patients with non-ACS should be discussed more seriously.

According to some studies, the frequency of no-reflow or SCF phenomenon in patients with ACS was significantly lower in patients who received intracoronary injection or intravenous drip of nicorandil.[8–10,19] The major difference between our study and recent studies is the method of nicorandil administration. Our simple method could also safely and effectively prevent SCF phenomenon. In 1999, Ito *et al.*[9] reported that intravenous nicorandil in conjunction with coronary angioplasty is associated with better functional and clinical outcomes in patients with an anterior AMI. In their protocol, nicorandil was administered by injection of 4 mg followed by infusion at 6 mg/h for 24 h and by oral administration. It is possible that the method was too complicated to be used routinely over the past 10 years. In 2003, Matsuo *et al.*[20] reported that intravenous injection of nicorandil 5 min before ballooning had a cardioprotective effect against ischaemia. In our protocol, administration of nicorandil was started 1 min before stent implantation because Okamura *et al.*[21] reported that intravenous administration of 6 mg of nicorandil increased APV and coronary blood flow, which were highest 30 s after the injection of nicorandil and were sustained at those levels for 4 min. Ishii *et al.*[8] reported that drip intravenous administration of 12 mg of nicorandil over a 20–30 min period before PCI led to beneficial clinical outcomes. We also tried to administer 12 mg of nicorandil intravenously for the first three patients in the preliminary study, but administration at this dose was stopped because systolic BP in the three patients decreased to levels much lower than expected after administration. Whereas their protocol of administration was intravenous drip over a 20–30 min period (0.4–0.6 mg/min), our protocol was intravenous bolus injection over a 20 s period (36 mg/min). Compared with intracoronary administration, we consider our method to be advantageous in a following point. In the case of total occlusion of the coronary artery, nicorandil could be delivered to the border area via small branches or collateral arteries.[22] In the J-WIND trial,[23] nicorandil administered intravenously failed to reduce infarct size or reperfusion injury. In that trial, nicorandil was administered intravenously at 0.067 mg/kg as a bolus, followed by 1.67 μg/kg/min as a 24 h continuous infusion. The dose of nicorandil administered as a bolus was less than that in our studies. It is possible that a higher dose might have been effective for reduction of infarct size in that trial.

Our method was useful for reduction of SCF phenomenon in both ACS and non-ACS patients. The Enhanced Myocardial Efficacy and Removal by Aspiration of Liberalized Debris trial failed to demonstrate a clear benefit of the routine use of distal protection during primary PCI compared with the conventional PCI strategy.[24] Previous studies have suggested that microcirculatory impairment is not solely caused by the distal embolization of atheromatous or thrombotic debris. Microcirculatory impairment is also thought to be caused by neutrophils by the direct effect of inflammatory mediators, including myeloperoxidase, elastase, and PR3, leading to myocardial damage.[25] We think that a balloon occlusion distal protection device cannot prevent the above-mentioned cells and humoral factors from flowing into distal microcirculation. On

Table 3 The rate of target vessel revascularization

	Total				ACS				Non-ACS			
	N (n = 177)	C (n = 163)	Relative risk	P-value	N (n = 39)	C (n = 46)	Relative risk	P-value	N (n = 138)	C (n = 117)	Relative risk	P-value
Total	21 (11.9)	28 (17.2)	0.65 (0.36–1.20)	0.17	4 (10.3)	14 (30.4)	0.27 (0.08–0.88)	0.04	17 (12.3)	14 (12.0)	1.03 (0.50–2.20)	1.00
Re-PCI	17 (9.6)	23 (14.1)	0.65 (0.34–1.26)	0.24	3 (7.7)	11 (23.9)	0.28 (0.08–1.10)	0.08	14 (10.1)	12 (10.3)	1.00 (0.44–2.23)	1.00
CABG	4 (2.3)	5 (3.1)	0.75 (0.19–2.77)	0.74	1 (2.6)	3 (6.5)	0.38 (0.04–3.78)	0.62	3 (2.2)	2 (1.7)	1.28 (0.21–7.78)	1.00

Table 4 Major adverse cardiac events for 12 months after percutaneous coronary intervention

	Total				ACS				Non-ACS			
	N (n = 185)	C (n = 179)	Relative risk	P-value	N (n = 40)	C (n = 48)	Relative risk	P-value	N (n = 145)	C (n = 131)	Relative risk	P-value
All deaths	12 (6.5)	12 (6.7)	0.97 (0.45–2.10)	0.93	3 (7.5)	5 (10.4)	0.72 (0.18–2.83)	0.72	9 (6.2)	7 (5.3)	1.16 (0.45–3.03)	0.76
Cardiovascular death	6 (3.2)	7 (3.9)	0.83 (0.28–2.42)	0.73	1 (2.5)	3 (6.3)	0.40 (0.04–3.70)	0.62	5 (3.4)	4 (3.1)	1.13 (0.31–4.12)	1.00
Admission for CHF	4 (2.2)	7 (3.9)	0.55 (0.16–1.86)	0.37	2 (5.0)	4 (8.3)	0.60 (0.12–3.11)	0.68	2 (1.4)	3 (2.3)	0.60 (0.10–3.55)	0.67

the other hand, previous studies have suggested that nicorandil reduces neutrophil infiltration into the ischaemic area.[26] Treatment combining intravenous administration of nicorandil and the use of a distal protection device may be more effective for preventing SCF or no-reflow phenomenon in PCI.

Study limitations

First, we measured serum CK and CK-MB levels of AMI patients to evaluate effects of nicorandil in acute phase. However, we could not assess enough about the infarct size only using serum maximum CK and CK-MB levels. Second, because there was no significant difference in the incidence rate of MACE for 12 months after PCI, further study with longer follow-up is required.

Conclusion

Intravenous administration of 6 mg of nicorandil immediately before PCI is a safe and simple procedure for preventing SCF phenomenon not only in patients undergoing emergent PCI for ACS but also in patients undergoing elective PCI for non-ACS. The rate of TVR was significantly lower in the nicorandil group than in the control group in ACS patients.

Conflict of interest: none declared.

References

1. Karagounis L, Sorensen SG, Menlove RL, Moreno F, Anderson JL. Does thrombolysis in myocardial infarction (TIMI) perfusion grade 2 represent a mostly patent artery or a mostly occluded artery? Enzymatic and electrocardiographic evidence from the TEAM-2 study. Second Multicenter Thrombolysis Trial of Eminase in Acute Myocardial Infarction. *J Am Coll Cardiol* 1992;**19**:1–10.
2. Ito H, Okamura A, Iwakura K, Masuyama T, Hori M, Takiuchi S, Negoro S, Nakatsuchi Y, Taniyama Y, Higashino Y, Fujii K, Minamino T. Myocardial perfusion patterns related to thrombolysis in myocardial infarction perfusion grades after coronary angioplasty in patients with acute anterior wall myocardial infarction. *Circulation* 1996;**93**:1993–1999.
3. Abbo KM, Dooris M, Glazier S, O'Neill WW, Byrd D, Grines CL, Safian RD. Features and outcome of no-reflow after percutaneous coronary intervention. *Am J Cardiol* 1995;**75**:778–782.
4. Kenner MD, Zajac EJ, Kondos GT, Dave R, Winkelmann JW, Joftus J, Laucevicius A, Kybarskis A, Berukstis E, Urbonas A, Feinstein SB. Ability of the no-reflow phenomenon during an acute myocardial infarction to predict left ventricular dysfunction at one-month follow-up. *Am J Cardiol* 1995;**76**:861–868.
5. Ito H, Maruyama A, Iwakura K, Takiuchi S, Masuyama T, Hori M, Higashino Y, Fujii K, Minamino T. Clinical implications of the 'no reflow' phenomenon. A predictor of complications and left ventricular remodeling in reperfused anterior wall myocardial infarction. *Circulation* 1996;**93**:223–228.
6. Rezkalla SH, Kloner RA. No-reflow phenomenon. *Circulation* 2002;**105**:656–662.
7. Kotani J, Nanto S, Mintz GS, Kitakaze M, Ohara T, Morozumi T, Nagata S, Hori M. Plaque gruel of atheromatous coronary lesion may contribute to the no-reflow phenomenon in patients with acute coronary syndrome. *Circulation* 2002;**106**:1672–1677.
8. Ishii H, Ichimiya S, Kanashiro M, Amano T, Imai K, Murohara T, Matsubara T. Impact of a single intravenous administration of nicorandil before reperfusion in patients with ST-segment-elevation myocardial infarction. *Circulation* 2005;**112**:1284–1288.
9. Ito H, Taniyama Y, Iwakura K, Nishikawa N, Masuyama T, Kuzuya T, Hori M, Higashino Y, Fujii K, Minamino T. Intravenous nicorandil can preserve microvascular integrity and myocardial viability in patients with reperfused anterior wall myocardial infarction. *J Am Coll Cardiol* 1999;**33**:654–660.
10. Sakata Y, Kodama K, Komamura K, Lim YJ, Ishikura F, Hirayama A, Kitakaze M, Masuyama T, Hori M. Salutary effect of adjunctive intracoronary nicorandil administration on restoration of myocardial blood flow and functional improvement in patients with acute myocardial infarction. *Am Heart J* 1997;**133**:616–621.
11. Austen WG, Edwards JE, Frye RL, Gensini GG, Gott VL, Griffith LS, McGoon DC, Murphy ML, Roe BB. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation* 1975;**51**(Suppl. 4):5–40.
12. Gibson CM, Cannon CP, Daley WL, Dodge JT Jr, Alexander B Jr, Marble SJ, McCabe CH, Raymond L, Fortin T, Poole WK, Braunwald E. TIMI frame count: a quantitative method of assessing coronary artery flow. *Circulation* 1996;**93**:879–888.
13. Antman EM, Giugliano RP, Gibson CM, McCabe CH, Coussement P, Kleiman NS, Vahanian A, Adgey AA, Menown I, Rupprecht HJ, Van der Wieken R, Ducas J, Scherer J, Anderson K, Van de Werf F, Braunwald E. Abciximab facilitates the rate and extent of thrombolysis: results of the thrombolysis in myocardial infarction (TIMI) 14 trial. The TIMI 14 Investigators. *Circulation* 1999;**99**:2720–2732.
14. Sato H, Iida H, Tanaka A, Tanaka H, Shimodouzono S, Uchida E, Kawarabayashi T, Yoshikawa J. The decrease of plaque volume during percutaneous coronary intervention has a negative impact on coronary flow in acute myocardial infarction: a major role of percutaneous coronary intervention-induced embolization. *J Am Coll Cardiol* 2004;**44**:300–304.
15. Piana RN, Paik GY, Moscucci M, Cohen DJ, Gibson CM, Kugelmass AD, Carrozza JP Jr, Kuntz RE, Baim DS. Incidence and treatment of 'no-reflow' after percutaneous coronary intervention. *Circulation* 1994;**89**:2514–2518.
16. Taniyama Y, Ito H, Iwakura K, Masuyama T, Hori M, Takiuchi S, Nishikawa N, Higashino Y, Fujii K, Minamino T. Beneficial effect of intracoronary verapamil on microvascular and myocardial salvage in patients with acute myocardial infarction. *J Am Coll Cardiol* 1997;**30**:1193–1199.
17. Taylor AJ, Al-Saadi N, Abdel-Aty H, Schulz-Menger J, Messroghli DR, Gross M, Dietz R, Friedrich MG. Elective percutaneous coronary intervention immediately impairs resting microvascular perfusion assessed by cardiac magnetic resonance imaging. *Am Heart J* 2006;**151**:891.e1–891.e7.
18. Korosoglou G, Geiger B, Hansen A, Hardt SA, Giannitsis E, Selter C, Katus HA, Kuecherer H. Usefulness of real-time myocardial perfusion imaging to evaluate alterations of myocardial blood flow in patients with stable angina pectoris undergoing elective percutaneous coronary interventions. *Am J Cardiol* 2005;**96**:885–891.
19. Lim SY, Bae EH, Jeong MH, Kang DG, Lee YS, Kim KH, Lee SH, Yoon KH, Hong SN, Park HW, Hong YJ, Kim JH, Kim W, Ahn YK, Cho JG, Park JC, Kang JC. Effect of combined intracoronary adenosine and nicorandil on no-reflow phenomenon during percutaneous coronary intervention. *Circ J* 2004;**68**:928–932.
20. Matsuo H, Watanabe S, Segawa T, Yasuda S, Hirose T, Iwama M, Tanaka S, Yamaki T, Matsuno Y, Tomita M, Minatoguchi S, Fujiwara H. Evidence of pharmacologic preconditioning during PTCA by intravenous pretreatment with ATP-sensitive K+ channel opener nicorandil. *Eur Heart J* 2003;**24**:1296–1303.
21. Okamura A, Rakugi H, Ohishi M, Yanagitani Y, Shimizu M, Nishii T, Taniyama Y, Asai T, Takiuchi S, Moriguchi K, Ohkuro M, Komai N, Yamada K, Inamoto N, Otsuka A, Higaki J, Ogihara T. Additive effects of nicorandil on coronary blood flow during continuous administration of nitroglycerin. *J Am Coll Cardiol* 2001;**37**:719–725.
22. Ishii H, Ichimiya S, Kanashiro M, Amano T, Matsubara T, Murohara T. Effects of intravenous nicorandil before reperfusion for acute myocardial infarction in patients with stress hyperglycemia. *Diabetes care* 2006;**29**:202–206.
23. Kitakaze M, Asakura M, Kim J, Shintani Y, Asanuma H, Hamasaki T, Seguchi O, Myoishi M, Minamino T, Ohara T, Nagai Y, Nanto S, Watanabe K, Fukuzawa S, Hirayama A, Nakamura N, Kimura K, Fujii K, Ishihara M, Saito Y, Tomoike H, Kitamura S. Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials. *Lancet* 2007;**370**:1483–1493.
24. Stone GW, Webb J, Cox DA, Brodie BR, Qureshi M, Kalynych A, Turco M, Schultheiss HP, Dulas D, Rutherford BD, Antoniucci D, Krucoff MW, Gibbons RJ, Jones D, Lansky AJ, Mehran R. Distal microcirculatory protection during percutaneous coronary intervention in acute ST-segment elevation myocardial infarction: a randomized controlled trial. *JAMA* 2005;**293**:1063–1072.
25. Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, Komatsu R, Ikura Y, Ogami M, Shimada Y, Ehara S, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 2002;**106**:2894–2900.
26. Mizumura T, Nithipatikom K, Gross GJ. Bimakalim, an ATP-sensitive potassium channel opener, mimics the effects of ischemic preconditioning to reduce infarct size, adenosine release, and neutrophil function in dogs. *Circulation* 1995;**92**:1236–1245.

Cell Metabolism

Angiopoietin-like Protein 2 Promotes Chronic Adipose Tissue Inflammation and Obesity-Related Systemic Insulin Resistance

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SUMMARY

Recent studies of obesity have provided new insights into the mechanisms underlying insulin resistance and metabolic dysregulation. Numerous efforts have been made to identify key regulators of obesity-linked adipose tissue inflammation and insulin resistance. We found that angiopoietin-like protein 2 (Angptl2) was secreted by adipose tissue and that its circulating level was closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans. Angptl2 activated an inflammatory cascade in endothelial cells via integrin signaling and induced chemotaxis of monocytes/macrophages. Constitutive Angptl2 activation in vivo induced inflammation of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased permeability. Angptl2 deletion ameliorated adipose tissue inflammation and systemic insulin resistance in diet-induced obese mice. Conversely, Angptl2 overexpression in adipose tissue caused local inflammation and systemic insulin resistance in nonobese mice. Thus, Angptl2 is a key adipocyte-derived inflammatory mediator that links obesity to systemic insulin resistance.

INTRODUCTION

Obesity is a pandemic medical and social problem that is associated with several adverse health outcomes, including type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, and cancer (Eckel et al., 2005; Mokdad et al., 2003), all of which result in increased mortality. A major metabolic manifestation of obesity is systemic insulin resistance. Recently, the concept has emerged that chronic low-grade activation of proinflammatory pathways in adipose tissue directly promotes systemic insulin resistance (Apovian et al., 2008; Neels and Olefsky, 2006; Schenk et al., 2008). Adipocytes and macrophages could be a source of several proinflammatory cytokines that activate inflammatory pathways in resident and infiltrating cells within adipose tissue in a paracrine or autocrine fashion (Kanda et al., 2006; Weisberg et al., 2006). However, the molecular mechanisms underlying inflammation of adipose tissue in obesity have not fully clarified.

Fibrinogen promotes leukocyte adhesion and cytokine secretion at sites of inflammation through integrin-dependent inflammatory pathways (Herrick et al., 1999; Mosesson, 2005).

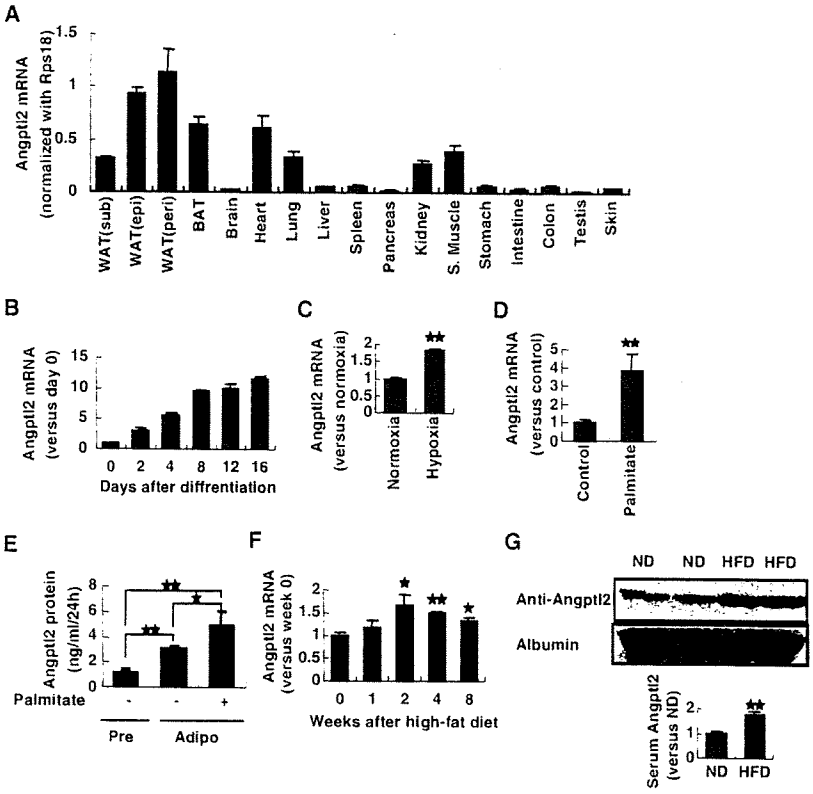

Figure 1. Angptl2 Is Secreted by Adipose Tissue

(A) Angptl2 mRNA expression in various tissues of mice fed normal chow (n = 4). WAT, white adipose tissue; sub, subcutaneous; peri, perirenal; mes, mesenteric; BAT, brown adipose tissue; S. Muscle, skeletal muscle.

(B–D) Angptl2 mRNA expression in 3T3-L1 cells during adipocyte differentiation (n = 3) (B), in differentiated 3T3-L1 cells incubated under hypoxic conditions (1% O_2, 24 hr, n = 3) (C), and in cells treated with palmitate (200 μM, 24 hr, n = 4) (D).

(E) Angptl2 protein levels in culture medium of pre- (Pre) or postdifferentiated (Adipo) 3T3-L1 cells with or without palmitate treatment (200 μM, 24 hr, n = 4).

(F) Angptl2 mRNA expression in the mesenteric adipose tissue of obese mice fed a high-fat diet for the indicated periods starting at 8 weeks of age (n = 4).

(G) Representative western blot and quantitative evaluation of serum Angptl2 protein in mice fed a normal diet (ND) or a high-fat diet (HFD) for a period of 8 weeks (n = 4). CBB-stained albumin is as control bands for protein loading. Data are the mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with controls.

Fibrinogen-binding integrins are abundantly expressed by monocytes/macrophages and endothelial cells, and fibrinogen must undergo oligomerization or polymerization to display its activity. The presence of extravascular fibrinogen at sites of inflammation has been documented by pathologists for decades (Dvorak et al., 1985). These findings prompted us to ask whether an oligomeric protein derived from adipose tissue and containing a fibrinogen-like sequence might play a pathological role in inflammatory changes of adipose tissue associated with obesity. Recently, we and others identified seven angiopoietin-like proteins (Angptls), which possess a coiled-coil domain at the N terminus for oligomerization and a C-terminal fibrinogen-like domain (Kim et al., 1999; Kubota et al., 2005a; Oike et al., 2004). Angptls are structurally similar to Tie-2 receptor ligands (angiopoietins), but Angptls do not bind to either Tie2 or the homologous Tie1 protein, indicating that their role differs from that of angiopoietins.

Here we show that angiopoietin-like protein 2 (Angptl2) is primarily secreted by adipose tissue and that its expression is increased by obesity and obesity-related pathological conditions, including hypoxia and endoplasmic reticulum (ER) stress. We found that increased circulating Angptl2 levels were closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans. Angptl2 acted on endothelial cells and monocytes/macrophages via integrin signaling, resulting in the promotion of inflammation. Constitutive activation of Angptl2 in mouse skin tissue induced chronic inflammation, including inflammatory changes of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased permeability. Deletion of Angptl2 led to reduced inflammation in adipose tissue and ameliorated systemic insulin resistance in mice with dietary obesity. Conversely, persistent overexpression of Angptl2 in adipose tissue caused local inflammation and systemic insulin resistance in nonobese mice. These findings establish Angptl2 as a key adipocyte-derived inflammatory mediator linking obesity to systemic insulin resistance and identify it as a new molecular target that could be used to improve the diagnosis and treatment of obesity and related metabolic diseases.

RESULTS

Angptl2 Expression in White Adipose Tissue Is Increased by Obesity and Obesity-Related Stress

Angptl2 mRNA was widely expressed in various organs of mice, but its level was particularly elevated in visceral white adipose tissues (Figure 1A). Differentiated 3T3-L1 adipocytes expressed Angptl2 mRNA (Figure 1B), and its expression was increased by hypoxia (Figure 1C), which occurs in obese adipose tissue (Hosogai et al., 2007; Nishimura et al., 2008; Schenk et al., 2008; Ye, 2009). We found significantly increased ER stress in adipocytes from obese mice compared with cells from nonobese mice (see Figure S1 available online). Serum levels of long-chain saturated fatty acids (LCSFAs) are elevated in obesity, and LCSFAs promote ER stress in adipocytes (Schenk et al., 2008). Our in vitro study of cultured 3T3-L1 cells revealed that ER stress was induced in adipocytes after treatment with palmitate, one of the LCSFA, or thapsigargin, an ER stress inducer. As